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Title: Update on Melatonin Receptors. IUPHAR Review

Short title: Melatonin Receptors

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Abstract

Melatonin receptors are seven transmembrane-spanning proteins belonging to the G protein-coupled receptor super-family. In mammals, two melatonin receptor subtypes exist MT₁ and MT₂ encoded by the *MTNR1A* and *MTNR1B* genes, respectively. The current review provides an update on melatonin receptors by the corresponding sub-committee of the International Union of Basic and Clinical Pharmacology. We will highlight recent developments of melatonin receptor ligands, including radioligands and give an update on the latest phenotyping results of melatonin receptor knockout mice. The current status and perspectives of the structure of melatonin receptor structures will be summarized. The physiological importance of melatonin receptor dimers and biologically important and type 2 diabetes-associated genetic variants of melatonin receptors will be discussed. The role of melatonin receptors in physiology and disease will be further exemplified by its functions in the immune system and the central nervous system. Finally, antioxidant and free radical scavenger properties of melatonin and its relation to melatonin receptors will be critically addressed.

Keywords (max. 10): GPCR oligomerization, sleep disorders, circadian rhythm disorders, depression, neuroprotection, genetic variants, type 2 diabetes, neurodegenerative diseases, free radical scavenger.

Abbreviations:

ADHD, attention-deficit/ hyperactivity disorder;

ASD, autism spectrum disorders;

AD, Alzheimer's disease;

BRET, bioluminescence resonance energy transfer;

CaMKII, Ca²⁺/Calmodulin (CaM)-dependent kinase II;

cAMP, cyclic adenosine monophosphate;

CNS, central nervous system;

ERG, electroretinogram;

fMLP, N-formyl-l-methionyl-l-leucyl-l-phenylalanine;

FPG; fasting plasma glucose;

GPCRs, G protein-coupled receptors;

HD, Huntington disease;

ICR, Imprinting Control Region;

IL-2, interleukin 2;

IOP, intraocular pressure;

IUPHAR, international union of basic and clinical pharmacology;

KO, knockout;

LPS, lipopolysaccharide;

MLT, melatonin;

MTR, melatonin receptor

MS, multiple sclerosis;

NREM, non-rapid eye movement;

PD, Parkinson disease;

REM, rapid eye movement;

SCN, suprachiasmatic nucleus;

QR2, quinone reductase 2;

T2D, type 2 diabetes.

Links

Targets

GPCRs^b	
5-HT2C	
β2-adrenergic receptor	
A2A adenosine receptor	
GPR50	
MT1 receptor	
MT2 receptor	
Channels	
Cav2.2	
Transporters^e	
GLUT1	
Enzymes^d	
CaMKII	
ERK1	

Ligands
6-chloromelatonin
[¹²⁵I]-DIV880
5-HEAT
6-hydroxymelatonin
2-iodomelatonin
2-[¹²⁵I]iodomelatonin
[³H]-melatonin
2-methoxy-α,β-didehydro-agomelatine
5-methoxyluzindole
4P-PDOT
[¹²⁵I]-SD6
[¹²⁵I]-S70254
agomelatine
BOMPPA
cAMP
CBOBNEA
CIFEA
Difluoroagomelatine

dopamine
EFPPEA
fMLP
GR 196429
GR 128107
(hydroxymethyl)phenyl agomelatine
IHK7IL-2
K185
Luzindole
MCA-NAT
N-acetyl-serotoninLPS
Melatonin
ramelteon
S22153
S24014
S24773
S26131
S26284
tasimelteon
TNF
TIK 301
UCM 454
UCM 765
UCM 793

Introduction

The hormone melatonin (MLT) is mainly produced by the pineal gland following a circadian rhythm, with high levels during the subjective night. MLT can also be produced by extra-pineal sites like the retina, the gastrointestinal tract and the innate immune system.

MLT regulates a variety of physiological and neuroendocrine functions through activation of two G protein-coupled melatonin receptors (MTR) called MT₁ and MT₂ in mammals. Both receptors are typically coupled to G_{i/o}-type proteins and MT₁ in addition to G_q-type proteins.

In humans, the *MTNR1A* gene encoding MT₁ is located on chromosome 4q35.1 and the

MTNR1B gene encoding MT₂ on chromosome 11q21-q22.

This article will review and discuss recent updates by the MTR sub-committee of the International Union of Basic and Clinical Pharmacology (IUPHAR) database (<http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=39>), which include the development of new MTR ligands, radioligands and structural perspectives of MTRs. The discovery of MTR dimers with physiological function *in vivo* as well as genetic variants and mutants of MTRs will be discussed as they provide a new dimension to understand MTR pharmacology and function. An update on the latest results obtained with MTR knockout (KO) mouse models will be provided. Among the multiple physiological effects of MTRs, we chose to focus on those of the immune system and the central nervous system (CNS). At the end, MTR-independent effects, including antioxidant and free radical scavenger properties of MLT will be critically addressed.

For more complete or other specific aspects of MTR the reader is referred to other recent expert reviews (Dubocovich *et al.*, 2010; Jockers *et al.*, 2008; Liu *et al.*, 2016; Markus *et al.*, 2013; Tosini *et al.*, 2014; Zlotos *et al.*, 2014).

Melatonin receptor ligands

MT₁ and MT₂ share a high degree of sequence homology and bind both the natural ligand, MLT, with high affinity. Important progress has been made in the identification of synthetic MTR antagonists and agonists and subtype-selective ligands by diversifying the chemical scaffolds. Indeed, MTR ligands from different structural classes show distinct structure activity relationships on native and recombinant MTRs (Dubocovich *et al.*, 2010; Dubocovich *et al.*, 1997; Spadoni *et al.*, 2011; Zlotos, 2012; Zlotos *et al.*, 2014). The methoxy group and the acetamido side chain of MLT determine the intrinsic activity and the binding affinity, respectively, at both hMT₁ and hMT₂ (Audinot *et al.*, 2003; Browning *et al.*, 2000; Dubocovich *et al.*, 1997). Replacement of the amide methyl group by ethyl and propyl

substituents enhances affinity (Sugden *et al.*, 1995). Exchange of the indole ring by various aromatic scaffolds maintains high binding and agonist potency.

Substitutions at the 2-position with a halogen or a phenyl group generate agonists with ~10-fold increased binding affinity. The majority of nonselective MT₁-MT₂ ligands, including drugs used in humans, i.e., **ramelteon** (Rozerem[®], (Kato *et al.*, 2005; Mini *et al.*, 2007; Rawashdeh *et al.*, 2011)), **agomelatine** (Valdoxan[®], (de Bodinat *et al.*, 2010)), and **tasimelteon** (Hetlioz[®], (Lavedan *et al.*, 2015; Rajaratnam *et al.*, 2009)), are agonists (Figure 1). Ramelteon and tasimelteon are MTR selective, while agomelatine is also an antagonist at the 5-HT_{2c} receptors, a pharmacological property believed to contribute to its antidepressant action. The therapeutic effects of approved drugs acting on hMT₁ and/or hMT₂ as agonists was recently reviewed (Liu *et al.*, 2016). Other nonselective MTR agonists include **6-chloromelatonin**, **6-hydroxymelatonin**, **2-iodomelatonin**, **GR 196429** (Audinot *et al.*, 2003; Browning *et al.*, 2000; Dubocovich *et al.*, 1997), **UCM 793** (Rivara *et al.*, 2007) and **2-methoxy- α,β -didehydro-agomelatine** (Morellato *et al.*, 2013). This latter ligand shows the highest affinity for hMT₁ ($K_i = 0.03$ nM) and hMT₂ ($K_i = 0.07$ nM) receptors and ~3,500-fold greater potency than MLT in the melanophore aggregation assay. **TIK 301** (Mulchahey *et al.*, 2004) acts also as an antagonist at the serotonin receptor subtypes 5-HT_{2c} and 5-HT_{2b}, (Landolt *et al.*, 2009). **5-HEAT** has a unique pharmacological profile acting as a full agonist at the hMT₁ and antagonist at the hMT₂ (Nonno *et al.*, 2000). **EFPEA**, a high-affinity hMT₁ ($K_i = 0.062$ nM) and hMT₂ ($K_i = 0.420$ nM) agonist, decreases the percentage of wakefulness and increases the percentage of slow wave sleep in cats (Koike *et al.*, 2011). The competitive MTR antagonist **luzindole** lacks the methoxy group, which led to the suggestion that this group is necessary for intrinsic activity (Dubocovich, 1988). Similarly, **S22153** acts as a partial agonist (Audinot *et al.*, 2003). Luzindole, with a 15- to 25-fold higher affinity for hMT₂ than for hMT₁, is widely used for pharmacological characterization of functional

MTRs (Browning *et al.*, 2000; Dubocovich *et al.*, 2010; Dubocovich *et al.*, 1997; Dubocovich *et al.*, 1998).

A ligand is considered selective for a specific receptor type when its affinity or potency is at least 100-times higher than that for the other(s) receptor types in the family (Dubocovich *et al.*, 2010). This concept holds true for *in vitro* studies where ligand concentrations can be easily adjusted. However, ligand selectivity might be more difficult to reach *in vivo*, in the body fluids reaching the receptors. Depending on ligand dose and pharmacokinetics, concentrations could easily raise to levels activating on both receptors (e.g., MT₁ and MT₂). This is particularly of concern for MLT and synthetic MTR ligands activating receptors at picomolar concentrations (Audinot *et al.*, 2003; Browning *et al.*, 2000; Dubocovich *et al.*, 1997). Therefore caution should be taken when interpreting selective MTR activation *in vivo* using MTR-selective ligands, unless pharmacological selectivity or lack of is confirmed by KO models with deletion of each receptor type.

Numerous ligands with high selectivity for hMT₂ over hMT₁ have been identified (Zlotos *et al.*, 2014). MT₂ possess a lipophilic pocket close to the N1–C2 binding region of MLT, which is absent in MT₁ (Rivara *et al.*, 2005). Accordingly, most MT₂-selective ligands bear a flexible bulky hydrophobic substituent in a position equivalent to C2 or N1 of MLT (Figure 2). The tetrahydroquinoline analogue **UCM1014** is the most potent MT₂-selective ligand reported to date. It shows picomolar binding affinity ($K_i = 0.001$ nM) at hMT₂, > 10,000-fold selectivity over hMT₁, and full agonist profile in the GTP γ S test (Spadoni *et al.*, 2015). Other agonists with approximately 800-fold hMT₂ selectivity are **BOMPPA** (Chan *et al.*, 2013; Heckman *et al.*, 2011; Hu *et al.*, 2013), and **CIFEAA** (Koike *et al.*, 2011). In imprinting control region (ICR) mice, **CIFEAA** reentrainment effects to a new light/dark cycle indicate the involvement of MTRs in the regulation of chronobiotic activity (Koike *et al.*, 2011). The dose of CIFEAA used in this study most likely reached μ M concentrations,

which would activate both MT₁ and MT₂, precluding any conclusion about the specific receptor type involved in the regulation of chronobiotic processes. Similarly, doses of the MT₂-selective antagonist 4P-PDOT (90 µg/mouse s.c.) used to block the MLT-mediated phase advance of circadian activity rhythms in mice (Dubocovich *et al.*, 1998) may have resulted in micromolar circulating 4P-PDOT concentrations hence blocking both MT₁ and MT₂.

Two moderately selective MT₂ ligands, the agonist **IIK7** (Faust *et al.*, 2000) and the partial agonist **UCM 765** (Rivara *et al.*, 2007) have been used to examine the role of each MTR type in the modulation of sleep architecture. **UCM 765** promoted non-rapid eye (NREM) movement sleep in rodents and this effect was blocked by the MT₂ antagonist **4P-PDOT** (Ochoa-Sanchez *et al.*, 2011). In contrast, the non-selective MT₁-MT₂ agonist **UCM793** decreased sleep onset without having an effect on NREM sleep maintenance suggesting that dual MT₁ and MT₂ agonistic activity accounts for the effect on sleep onset, whereas selectivity for MT₂ has an additional effect on NREM sleep maintenance. **IIK7** was also reported to reduce NREM sleep onset latency and transiently increase the time spent in NREM sleep in rats without altering REM sleep latency or the amount of REM sleep (Fisher *et al.*, 2009).

Among the hMT₂-selective partial agonists **GR 128107**, **5-methoxyluzindole**, **S 24014**, **S 24773** (Audinot *et al.*, 2003; Dubocovich *et al.*, 1997), and **isoamyl agomelatine**, the latter shows the highest affinity ($K_i = 0.01$ nM) and selectivity (7,200-fold) (Ettaoussi *et al.*, 2012). **4P-PDOT**, an hMT₂-selective antagonist with 300- to 1,500-fold higher affinity for hMT₂, is still considered the gold standard for pharmacological characterization of MTRs (Dubocovich *et al.*, 1997). Other MT₂-selective antagonists, such as **K185** (Faust *et al.*, 2000; Sugden *et al.*, 1999), **UCM 454** (Rivara *et al.*, 2005), and **2-(indolin-1yl) melatonin** (Zlotos *et al.*, 2009) display ~100-fold higher affinity for hMT₂. For **(hydroxymethyl)phenyl**

agomelatine, the affinity for hMT₂ is 750-times higher than for hMT₁ (Poissonnier-Durieux *et al.*, 2008).

Discovery of MT₁-selective ligands remains a challenge, and only few compounds with preference for hMT₁ have been reported (Zlotos *et al.*, 2014). Ligands preferentially binding to hMT₁ reach maximally 100-fold selectivity, and, when investigated, this selectivity is significantly reduced in functional *in vitro* studies (Figure 3). A common structural feature conferring MT₁ selectivity is a bulky, hydrophobic ether replacing the methoxy group. The first hMT₁-selective agents were obtained by connecting two agomelatine units via their ether oxygens by (CH₂)₃- and (CH₂)₄-linker to give **S 26131** (antagonist) and **S 26284** (partial agonist), both displaying ~100-fold selectivity (Audinot *et al.*, 2003; Descamps-Francois *et al.*, 2003). A similar approach led to the **UCM 793 dimer** with 100-fold hMT₁ selectivity and partial agonist activity (Spadoni *et al.*, 2011). Monomeric ligands, such as **CBOBNEA** (Mèsangeau *et al.*, 2010), and **AAE M PBP amine** (Rivara *et al.*, 2012) are partial agonists showing similar, ~100-fold hMT₁ selectivity. **N-Acetyl-O-phenoxypropyl serotonin** is a full agonist obtained by exchange of the methoxy group of MLT with an O(CH₂)₃OPh moiety. Although it shows only 10-fold binding preference toward hMT₁, its MT₁-MT₂ binding ratio and hMT₁ affinity were higher than that for the MT₁-selective reference **S 26131** that was retested under the same experimental conditions (Markl *et al.*, 2011). A 140-fold MT₁ selectivity could be accomplished by introduction of two fluorine atoms into the *N*-acetyl group of agomelatine. The resulting **difluoroagomelatine** shows high hMT₁ binding ($K_i = 0.03$ nM), and is a nonselective MT₁-MT₂ full agonist (Ettaoussi *et al.*, 2012). Very recently, **tetrafluoro S26131, the difluoroacetamide analogue of S 26131**, has been reported to show higher affinity and selectivity toward hMT₁ than the parent ligand (Zlotos *et al.*, 2015).

In summary, while numerous MTR ligands selective for the MT₂ subtype are available, discovery of ligands with at least >100-fold selectivity for MT₁ remains a challenging task. None of the MT₁-selective ligands has been tested *in vivo*. Future progress on the elucidation of the structure of MTRs will hopefully foster the discovery of such ligands. The MT₁-MT₂ nonselective receptor antagonist **luzindole** and the MT₂-selective antagonists **4P-PDOT** are still considered the gold standards for pharmacological characterization of MTRs.

Radioligands - update

Radioactive- and fluorescent-labeled ligands are indispensable tools for the pharmacological characterization of G protein-coupled receptors (GPCRs). A major breakthrough in the field of MTR research was the labelling of 2-iodomelatonin with ¹²⁵I at carbon 2 resulting in a high affinity radioligand with high specific activity (Vakkuri *et al.*, 1984) for use in the localization (Vanecek, 1988) and pharmacological characterization of MTR in tissues (Dubocovich *et al.*, 1987). The radioligand, 2-[¹²⁵I]iodomelatonin (2-[¹²⁵I]-MLT), has been extensively used as a high-affinity radioligand for both MT₁ and MT₂, which was until now the only available radioligand for the characterization and localization of MLT binding sites in native tissues (Figure 4). Studies performed with [³H]-melatonin ([³H]-MLT) established the pharmacological profile of the human recombinant MT₁ and MT₂, as being identical to that established using 2-[¹²⁵I]-MLT as a radioligand. However due to the rather low specific activity of this ³H-MLT its use to characterize and/or localize MLT sites in tissues with low MTR density is hampered (Browning *et al.*, 2000).

Three new iodinated radioligands have been recently characterized for use in the pharmacological characterization and localization of MTRs (Figure 4). These radioligands are: SD6 (N-[2-(5-methoxy-1H-indol-3-yl)ethyl]iodoacetamide), S70254 (2-iodo-N-2-[5-methoxy-2-(naphthalen-1-yl)-1H-pyrrolo[3,2-b]pyridine-3-yl]acetamide and DIV880 2-(2-[(2-iodo-4,5-dimethoxyphenyl)methyl]-4,5-dimethoxy phenyl) (Legros *et al.*, 2016; Legros *et al.*, 2013). [¹²⁵I]-SD6 has a similar pharmacological profile than 2-[¹²⁵I]-MLT with the same affinity for MT₁ and MT₂. On the contrary, the two other radioligands [¹²⁵I]-S70254 and [¹²⁵I]-DIV880 show selectivity for MT₂ with pK_d values of 9.6 and 9.7, respectively, in the absence of any specific binding to MT₁. All radioligands are agonists, either partial agonists ([¹²⁵I]-S70254, [¹²⁵I]-DIV880) or full agonists (2-[¹²⁵I]-MLT, [¹²⁵I]-SD6, [³H]-MLT) which means that their K_d values not only depend on the affinity of the ligand for the receptor but also on the activation of the G protein in the ternary Ligand-Receptor-G protein complex.

The extensive pharmacological characterization of these three new radioligands in comparison with 2-[¹²⁵I]-MLT and [³H]-MLT on membrane preparations from CHO-K1 cell lines stably expressing hMT₁ or hMT₂ showed that [¹²⁵I]-S70254 and [¹²⁵I]-DIV880 mainly differ from 2-[¹²⁵I]-MLT in its dissociation kinetics, which are faster for [¹²⁵I]-S70254 and [¹²⁵I]-DIV880 than for 2-[¹²⁵I]-MLT (Legros *et al.*, 2016). Interestingly, [¹²⁵I]-SD6 labeled only approximately half of the binding sites detected with 2-[¹²⁵I]-MLT in cells expressing hMT₁ while comparable amounts were detected in cells expressing hMT₂ (Legros *et al.*, 2013). This suggests the existence of different receptor sub-populations for hMT₁ of which [¹²⁵I]-SD6 labels a more restricted number than 2-[¹²⁵I]-MLT. In contrast, for hMT₂ similar sub-populations would be detectable by both radioligands. The nature of these receptor sub-populations is currently unknown but could be related to the differential engagement of hMT₁ into complexes with different G proteins or β-arrestins following the binding of these

agonistic radioligands. [^{125}I]-SD6 detected as 2-[^{125}I]-MLT binding sites in sheep retinal membranes, while the MT₂-specific ligands [^{125}I]-S70254 and [^{125}I]-DIV880 failed to do so.

The MT₂-specific [^{125}I]-S70254 was successfully used for autoradiography studies in rat and sheep brain and retina slices (Legros *et al.*, 2016). A similar labeling pattern to 2-[^{125}I]-MLT (detecting MT₁ and MT₂) was observed in several areas but also distinct labeling in others. Absence of labeling by [^{125}I]-S70254 in regions that are labeled by 2-[^{125}I]-MLT can be explained by low(undetectable) MT₂ expression levels. Absence of 2-[^{125}I]-MLT labeling in regions labeled by [^{125}I]-S70254 could be due to the detection of different receptor complexes (see above).

Altogether, the new radioligands considerably expand the repertoire of pharmacological tools for MTRs with the development of MT₂-specific radioligands and radioligands detecting distinct receptor populations revealing a previously unrecognized diversity. The availability of a radiolabeled antagonist would largely contribute in a better characterization of these different populations. Further advances can be expected from the development of fluorescent-labeled ligands.

Structural perspectives for melatonin receptors

Currently, crystal structures of MT₁ and MT₂ are not available. Despite a sequence identity lower than 30% between MTRs and the closest crystallized GPCRs, several 3D models have produced some structural hypothesis for binding of (non)selective MT₁ or/and MT₂ agonists (Table I). According to site-directed mutagenesis data, most of these models corroborate the importance of both serine residues 3.35 and 3.39 in MT₁ as well as His5.46 in both MT₁ and MT₂. Although His5.46 seems to be an anchoring residue for polar interactions with the methoxy or amide group of MLT, only a few MT₁ models display a direct

participation of serine residues in MLT binding (Chugunov *et al.*, 2006; Farce *et al.*, 2008), which could be otherwise involved in an essential bending of helix 3 for binding site plasticity. Models take also into account several receptor-ligand interactions with amino acids conserved within GPCRs and known to play a role in aromatic switch activation (F5.47, W6.48).

Such homology modelling methods make predictions of flexible receptor regions difficult. Although not directly proven for MTRs, E2 and I3 loops are known to be key features for ligand accessibility and G protein binding of GPCRs. Moreover amino acid sequences of MTRs show several singularities like the presence of a ^{3.49}NR^{3.51}Y motif instead of the classical ^{3.49}DR^{3.51}Y motif of other rhodopsin-like GPCRs. Another specificity is the replacement of the proline by an alanine residue in the conserved ^{7.49}NPXX^{7.53}Y motif.

Buried in the vicinity of the cytoplasmic surface, these marked differences are likely to impact on receptor activation and/or signalling specificity of MTRs rather than the ligand binding process.

Whereas 3D models of MTRs were up to now dedicated to the discovery and optimization of new efficient drugs, the next generation of 3D models should be expanded towards larger, multimeric systems and not be restricted to receptor monomers. Computation of the energy landscape of GPCRs by enhanced molecular dynamics simulations, together with NMR and X-ray studies, provided valuable molecular insights on the dynamics of ligand recognition, receptor activation and oligomerization (Johnston *et al.*, 2014). Depicting free energy landscapes of MTRs should address biasing molecular dynamics simulations from the inactive apoform transiting toward the active trimeric L-R-G_i or L-R-arrestin forms of receptors (Figure 5). As ligands modulate these free energy landscapes (Dror *et al.*, 2013; Provasi *et al.*, 2011), *in silico* optimization of new efficient ligand structures could be explored by predicting its functional selectivity through arrestin or G_i-mediated pathways.

These approaches also open the way for the exploration of homo- and heterodimers, particularly MT₁/MT₂ and MT₁/GPR50 complexes, as discussed in the following section.

Melatonin receptor dimers

MTRs are part of dynamic signaling complexes that contain proteins involved in receptor biosynthesis, export, signaling, desensitization, internalization and cytoskeleton modulation (Daulat *et al.*, 2007; Maurice *et al.*, 2008) (IntAct database, <http://www.ebi.ac.uk/intact/search/do/search?searchString=pubid:26514267>). The core of these complexes is often composed of receptor dimers, either homodimers of the same receptor or heterodimers composed of two different receptors (see (Ferre *et al.*, 2014; Maurice *et al.*, 2011) for review). Initial observations have been made in 2002 in transfected HEK293 cells demonstrating the capacity of MT₁ and MT₂ to form homo- and heterodimers (Ayoub *et al.*, 2002) with MT₁/MT₂ heterodimers showing a pharmacological profile distinct from MT₂ homodimers (Ayoub *et al.*, 2004). Shortly after, MT₁ and MT₂ were reported to form heterodimers with the orphan GPR50, which completely abolished the function of MT₁ in MT₁/GPR50 heterodimers (Levoye *et al.*, 2006a). Sporadic reports on Western blots with endogenously expressed MTRs in chicken astrocyte cultures (Adachi *et al.*, 2002) and *Xenopus* tectal cells (Prada *et al.*, 2005) further indicated the possible existence of MTR homodimers. However, the physiological relevance of these dimers remained largely unclear (Levoye *et al.*, 2006b) until 2013 when compelling *in vivo* evidence for the functional significance of MT₁/MT₂ heterodimers was obtained. In retinal photoreceptor cells, MLT enhances the light sensitivity during the night. The phenotype of MT₁ KO (MT₁^{-/-}) and MT₂ KOs (MT₂^{-/-}) mice, the use of type-selective ligands and over-expression of a dominant negative form of MT₂ in photoreceptor cells of transgenic mice indicated the exclusive involvement of MT₁/MT₂ heterodimers in this physiological effect of MLT (Baba *et al.*,

2013). Interestingly, this effect was dependent on the activation of the G_q/PLC pathway by MLT, an observation that could be confirmed *in vitro* in cells co-expressing MT₁ and MT₂.

Whether MTR heterodimers could become novel drug targets remains an open question. Recent evidence on the antidepressant agomelatine suggests this possibility (Kamal *et al.*, 2015). Previous studies showed that agomelatine is a high affinity agonist for MT₁ and MT₂ and an antagonist with moderate affinity for serotonin 5-HT_{2C} receptors (Audinot *et al.*, 2003; Millan *et al.*, 2003). Of note, the antidepressant effect of agomelatine involves both pathways in a synergistic manner (Racagni *et al.*, 2011). Formation of MT₂/5-HT_{2C} heterodimers was demonstrated in transfected HEK293 cells and these heterodimers are targeted by agomelatine (Kamal *et al.*, 2015). Agomelatine behaved as a biased ligand, activating the G_i/cAMP pathway and antagonizing the G_q/PLC pathway. Whether the MT₂/5-HT_{2C} heterodimer participates in the antidepressant effect of agomelatine remains to be shown.

Formation of receptor dimers offers the possibility to design dimeric ligands targeting receptor dimers. Several dimeric ligands with two identical pharmacophores have been synthesized for MTRs and their binding properties have been determined (Audinot *et al.*, 2003; Descamps-Francois *et al.*, 2003; Journe *et al.*, 2014; Mesangeau *et al.*, 2010; Spadoni *et al.*, 2011). Binding of the two pharmacophores of these dimeric ligands to the two protomers of the same receptor dimer has been only shown in one study using a bioluminescence resonance energy transfer (BRET) approach (Journe *et al.*, 2014). Compounds linked through 22–24 atom spacers were able to bind to MT₁ and MT₂ protomers in pre-existing homo- and heterodimers and to induce conformational changes detected by BRET. Induction of receptor dimerization was not observed. The functional properties of these compounds remain to be studied. Taken together, the existence and physiological relevance of MTR dimers is increasingly recognized but its functional role and

pharmacological exploitation are still ongoing.

Genetic variants and mutants of melatonin receptors

The existence of multiple rare variants in the human population was discovered in recent genome sequencing programs. The 1000 human genome project detected 38 million variants (Abecasis *et al.*, 2012) and 172 variants, including 46 non-synonymous variants, have been identified on average per GPCR in a population of 14002 individuals (Karamitri *et al.*, 2014; Nelson *et al.*, 2012). Numerous variants have been identified in the *MTNRIA* and *MTNR1B* genes, encoding MT₁ and MT₂ receptors, respectively. Here, only non-synonymous variants, modifying the amino acid sequence of the receptors, will be considered (Figure 6). Variants with altered receptor function can potentially participate in disease development.

Ebisawa *et al.* (1999) were searching for variants in *MTNRIA* and *MTNR1B* genes in patients with circadian disorders. Two non-synonymous variants were identified in the *MTNRIA* gene (R54W, A157V) which were 3-fold and 2-fold more frequent in people with non-24-hour sleep-wake syndrome (Table II) (Ebisawa *et al.*, 1999). Due to small sample size (N=22) statistical significance was not reached.

Alteration of MLT synthesis has been reported in autism spectrum disorders (ASD) triggering the search for variants in *MTNRIA* and *MTNR1B* genes in 295 patients with ASD, 362 controls and 284 individuals from the human genome diversity panel (Chaste *et al.*, 2010). Six non-synonymous mutations were identified for *MTNRIA* and ten for *MTNR1B* (Table II, III). The majority of these mutants showed altered receptor function. Particularly deleterious mutants were MT₁-I49N, which is devoid of any MLT binding and cell surface expression, and MT₁-G166E and MT₁-I212T, which showed severely impaired cell surface expression and biased behavior towards the ERK1/2 pathway. No significant difference in the

prevalence of these mutations was found indicating that they do not represent major risk factor for ASD.

Four non-synonymous mutations were identified for *MTNR1A* and four for *MTNR1B* in a cohort of 101 individuals with attention-deficit/ hyperactivity disorder (ADHD) (Table II, III), however, none of them was enriched in ADHD individuals as compared to the general population (Chaste *et al.*, 2011). The MT₁-Y170X non-sense mutation was only detected in one ADHD patient and introduced a premature STOP codon resulting in complete loss of receptor function.

MTR variants have been most extensively searched in studies focused on type 2 diabetes (T2D) based on the discovery of several frequent polymorphisms associated with increased fasting plasma glucose (FPG) and T2D risk close to the *MTNR1B* gene in genome-wide association studies (Bouatia-Naji *et al.*, 2009; Prokopenko *et al.*, 2009). Sequencing of the coding region of the *MTNR1B* gene revealed six non-synonymous variants (G24E, L60R, V124I, R138C, R231H, K243R) of which none was associated with T2D risk. The common 24E variant was associated with increased body mass and decreased FPG (Andersson *et al.*, 2010), an observation that was not replicated in a later study (Bonfond *et al.*, 2012). Whereas only subtle changes in the capacity of G24E and V124I to activate a G α Δ 6qi4myr chimeric G protein, the L60R variant was completely inactive in transfected COS cells. A more extensive sequencing study discovered 40 non-synonymous variants in the coding region of *MTNR1B* (Table II, III, IV) (Bonfond *et al.*, 2012) of which 36 very rare mutants associated with T2D risk. Functional analysis of the 40 variants revealed intact cell surface expression for all variants, complete loss of MLT binding in 4 very rare cases (A42P, L60R, P95L, Y308S) and partially and severely blunted signaling (G α qi9 chimera and ERK1/2 activation) in 1 rare cases (R138C) and 9 very rare cases (W22L, A52T, A74T, R138H,

R138L, L166I, R222H, R330W, I353T). Carriers of the 13 very rare loss-of-function variants showed increased T2D risk establishing a functional link between *MTNR1B* and T2D (for review see (Karamitri *et al.*, 2013)).

In conclusion, the genetic variability of the *MTNR1B* gene in terms of non-synonymous variants has now been well defined and an association of very rare variants with T2D risk established. Less is known about the variability of the *MTNR1A* gene in terms of non-synonymous variants.

Melatonin receptor mouse models - update

MT₁^{-/-} mice were created in the late 90s' followed by the generation of MT₂^{-/-} mice in 2003 (Jin *et al.*, 2003; Liu *et al.*, 1997). Studies using these mice have provided important insights on the role that MTRs play in the modulation of many different biological functions. In MT₁^{-/-} mice, but not in MT₂^{-/-} mice, the inhibitory effect of MLT on neuronal activity in the suprachiasmatic nucleus (SCN) is impaired suggesting the involvement of MT₁. In contrast, in SCN slices from MT₁^{-/-} mice MLT (1-10 pM) phase-shifts the peak of circadian rhythms of neuronal firing by approximately 3 h suggesting the involvement of MT₂ (Dubocovich *et al.*, 2005; Liu *et al.*, 1997). Blockade of this effect using the MT₂ selective 4P-PDOT antagonist confirmed the latter conclusion shaping a pathway where MT₂ phase-shifts the peak of neuronal firing through PLC-PKC signaling pathway (Dubocovich *et al.*, 2005; Hunt *et al.*, 2001) (Mc Arthur *et al.*, 1997). Liu *et al.* (1997) reported that the phase shift of neuronal firing rhythms induced by 2-iodomelatonin (10 pM) was of smaller magnitude in the SCN slice from MT₁^{-/-} than in WT mice suggesting a role for the MT₁ in this response (see detailed discussion in (Dubocovich, 2007)). Together these findings suggest a potential role for both MT₁ and MT₂ in the phase shift of circadian rhythms of neuronal firing in the SCN slice *in vitro*. The use of MT₁^{-/-} mice demonstrated that MT₁ is required for the MLT-mediated

phase-shift of the onset of overt circadian rhythm of locomotor activity (Dubocovich *et al.*, 2005). An independent study demonstrated that C3H/HeN mice (MLT-proficient) entrained faster to a phase advance of dark onset than the C57BL/6J mice (MLT-deficient), suggesting a facilitating role of endogenous MLT on circadian reentrainment (Pfeffer *et al.*, 2012). However, we should note that faster entrainment could also result from genetic differences between the two mouse strains rather than different endogenous melatonin levels (Adamah-Biassi *et al.*, 2013). In a mice strain producing endogenous melatonin the faster entrainment to an abrupt advance of dark onset persisted in $MT_1^{-/-}$ C3H/HeN mice but was lost in $MT_2^{-/-}$ and double KOs ($MT_1^{-/-}/MT_2^{-/-}$) suggesting again the involvement of MT_2 . This apparent contradiction could be explained by the activation of MT_2 and MT_1 by endogenous and exogenous MLT, respectively, at different periods of sensitivity (subjective night vs. subjective day, respectively). Changes in efficacy could also result from desensitization and/or internalization of MTRs in response to exposure to physiological and supraphysiological MLT concentrations as demonstrated by the phase shift of the peak of neuronal firing in the SCN by physiological levels of MLT, which involved the desensitization of MT_2 (Gerdin *et al.*, 2004).

MTR KO mice have been also used to elucidate the role played by these receptors in the regulation of the sleep/wake cycle. In $MT_2^{-/-}$ NREM sleep is decreased during the light phase (i.e., during the time that mice normally sleep), whereas $MT_1^{-/-}$ mice showed an increase in the amount of NREM sleep during the dark phase (i.e., during active phase) (Ochoa-Sanchez *et al.*, 2011). Further analysis of the data indicated that MT_1 signaling is implicated in the modulation of the daily rhythm of REM sleep (Ochoa-Sanchez *et al.*, 2011). An additional study in which double KOs ($MT_1^{-/-}/MT_2^{-/-}$) were used indicated that removal of both receptors induced an increase in wakefulness, and a reduction in REM sleep (Comai *et*

al., 2013). Hence these data seem to indicate that removal of MTRs may affect wakefulness rather than sleep.

The effect of MTR removal has been also investigated in the mouse retina, where these receptors are widely distributed (Baba *et al.*, 2013; Baba *et al.*, 2009). Removal of either receptor has profound effects on photoreceptors function as it abolishes the daily rhythms in the scotopic and photopic electroretinogram (ERGs) (Alcantara-Contreras *et al.*, 2011; Baba *et al.*, 2009; Sengupta *et al.*, 2011). Such a result also indicates that MT₁ and MT₂ form heterodimers in mouse photoreceptors (Baba *et al.*, 2013). Further studies have also demonstrated that removal of MTRs in addition affects the viability of the photoreceptors and retinal ganglion cells during aging (Alcantara-Contreras *et al.*, 2011; Baba *et al.*, 2009; Giancesini *et al.*, 2016) as well as corneal biology (Baba *et al.*, 2015).

As mentioned before, recent studies have also implicated MTRs in the pathogenesis of T2D in humans (Bonfond *et al.*, 2012; Bouatia-Naji *et al.*, 2009; Lyssenko *et al.*, 2009). Thus a few studies used MTR KO mice to determine the mechanisms by which these receptors contribute to regulation of glucose homeostasis and insulin sensitivity (Contreras-Alcantara *et al.*, 2010; Muhlbauer *et al.*, 2009; Stumpf *et al.*, 2008). Mice lacking MT₁ exhibit higher mean blood glucose levels than controls (Muhlbauer *et al.*, 2009), and tend to be more glucose intolerant and insulin resistant than WT and MT₂^{-/-} mice (Contreras-Alcantara *et al.*, 2010). Furthermore, removal of MT₁ or MT₂ abolishes the daily rhythm in blood glucose levels (Owino *et al.*, 2016).

Finally, it is important to mention that although the reproductive system of mice is not sensitive to photoperiod, the development of MTR KO mice provided an important tool for dissecting the mechanisms by which MLT regulates reproduction in photoperiodic species. For example, MT₁ signaling controls the rhythmic expression of the clock gene *Period 1* in the pituitary gland (von Gall *et al.*, 2002) and further studies have shown that the rhythmic expression of several other clock genes (*Per1*, *Per 2*, *Bmal1*, and *Cry 1*) in the mouse *Pars tuberalis* depends on MT₁ signaling as well (Jilg *et al.*, 2005). MT₁ signaling has been also reported to be crucial for the photoperiodic response of gene expression in the ependymal cell layer and thus for the photoperiodic regulation of gonadal activity (Sheynzon *et al.*, 2006; Yasuo *et al.*, 2009). Finally, we should mention that a recent study reported that MT₁ signaling plays a key role in photoperiodic programming of serotonergic neurons as well as depression- and anxiety-related behaviors in mice (Green *et al.*, 2015).

In conclusion studies in the last twenty years using MTR KO mice have greatly helped to understand the role(s) played by these receptors in the regulation of many physiological functions and they have provided important insights on the mechanisms by which MLT signaling affects these functions.

Functional role of melatonin receptors in physiology and pathophysiology

MTRs are involved in many physiological processes that will however not all be covered by this review but can be consulted in other reviews (Dubocovich *et al.*, 2010; Johnston *et al.*, 2015; Karamitri *et al.*, 2013; Tosini *et al.*, 2012; Tosini *et al.*, 2014). Here, we focused our attention on two major systems, the immune and the central nervous system. Important progress has been made recently in both fields and links to diseases have been established justifying a review of our current knowledge on these aspects. Finally, we will make a critical assessment of reports of receptor-independent effects of MLT, such as binding

of MLT to additional binding site and intrinsic antioxidant and free radical scavenger properties of MLT.

- Melatonin receptors in the immune system

The role of MLT as a player in immunity, first proposed by Berman in 1926, is now well accepted (Carrillo-Vico *et al.*, 2013). Several reports demonstrated that MLT produced either by the pineal gland or immune cells can regulate the activation of an immune response. MLT derived from activated human lymphocytes induces the synthesis of interleukin 2 (IL-2) and IL-2 receptors ((Carrillo *et al.*, 2004; Carrillo-Vico *et al.*, 2013). Luzindole and targeted deletion of the *MNTR1A* gene (Lardone *et al.*, 2006; Lardone *et al.*, 2010) block the effect of lymphocyte-derived MLT. Interestingly, daily rhythms of plasma MLT and IL-2 are transiently lost in non-infectious human inflammatory conditions and the recovery of the IL-2 rhythm follows the restoration of the daily MLT rhythm (Pontes *et al.*, 2007). In addition, the daily and seasonal variation of MLT production contributes to the seasonality of some diseases. In multiple sclerosis (MS) MLT blocks the differentiation of Th17 cells and boosts the generation of protective Tr1 cells by a MT_1 -dependent mechanism, resulting in the seasonal variation on MS symptoms (Farez *et al.*, 2015). Seasonality of regular immunity is also related to changes in the MLT system (Weil *et al.*, 2015). In the spleen of several species extended light exposure decreases MT_1 expression (Lahiri *et al.*, 2009; Maestroni, 1993; Yadav *et al.*, 2013). In healthy conditions rolling and adhesion of neutrophils to the endothelial cell layer is inhibited by activation of MT_2 and ligands binding to the putative MT_3 binding site, respectively (Lotufo *et al.*, 2001). In contrast, other effects of MLT such as the inhibition of transcription factors that mediate acute inflammation induced by lipopolysaccharides (LPS) (Tamura *et al.*, 2010) or N-formyl-l-methionyl-l-leucyl-l-

phenylalanine (fMLP) (Cernysiov *et al.*, 2015) are not blocked by luzindole suggesting a MTR-independent action mode.

MTRs also play an important role in promoting engulfing of bacteria, fungi and parasites. MLT facilitates the invasion of erythrocytes by *Plasmodium falciparum* (Hotta *et al.*, 2000) the invasion of macrophages by *Leishmania amazonensis* (Laranjeira-Silva *et al.*, 2015) and the phagocytosis of zymosan by colostrum polymorphonuclear and mononuclear cells (Pires-Lapa *et al.*, 2013) and the RAW 264.7 macrophage cell line (Muxel *et al.*, 2012). The entrance of different microorganisms in polymorpho and monuclear cells, including colostrum and lineage established cell lineages is blocked by luzindole. Indeed, parasites, bacteria and fungi activate the NF- κ B pathway in these two cell models resulting in the expression of arylalkyl-N-acetyltransferase and the synthesis of MLT. Luzindole and 4P-PDOT blocked the expression of dectin-1, a protein that is important for phagocytosis, suggesting the participation of MT₂ in this effect (Muxel *et al.*, 2016; Muxel *et al.*, 2012; Pires-Lapa *et al.*, 2013). Thus, the evaluation of binding parameters and functional states of MTRs in immune competent cells need to consider the masking effect of on-demand synthesized MLT.

Although complex, the role of MLT on the immune system is beginning to be understood. MT₁ and MT₂ receptor types appear to play different roles, MT₁ is the main target in acquired immune response, and MT₂ the target for innate immune responses.

- Melatonin receptors in the CNS

MTRs are widely expressed throughout the CNS, and are particularly well characterized in the SCN of the hypothalamus, where they are known to inhibit neuronal firing and mediate the phase-shifting effect of MLT on circadian rhythms (see above). In addition to its chronobiotic effect, MLT participates in the modulation of neuronal functions,

neurodevelopment at early and late stages (Kong et al., 2008; Chen et al., 2014) and affects brain structures underlying sleep regulation (Ochoa-Sanchez *et al.*, 2011), drug-related learning (Savaskan *et al.*, 2006; Wang *et al.*, 2005) and reward (Clough *et al.*, 2014; Hutchinson *et al.*, 2012). MTRs have been shown to mediate the MLT-induced increase in dendrite length, thickness and complexity of hippocampal neurons, as these effects were partially blocked by luzindole (Dominguez-Alonso *et al.*, 2015). Similarly, MLT-induced differentiation and maturation of adult neural stem cells was almost abrogated in the presence of luzindole (de la Fuente Revenga *et al.*, 2015). A recent study using $MT_2^{-/-}$ mice reveals that MT_2 is essential for axogenesis and for the formation of functional synapses (Liu *et al.*, 2015). MT_2 is also involved in MLT-induced protection against oxidative stress and memory impairment in a mice model of aging (Shin *et al.*, 2015). Recent advances in the understanding of presynaptic MTRs and their role in neurodegenerative diseases are discussed in the following chapters.

Presynaptic melatonin receptors. The role of MLT on the regulation of calcium-dependent dopamine release from axon terminals in brain and amacrine cells in the retina was shown in the early 1980's (Dubocovich, 1983; Zisapel *et al.*, 1982). However, more direct and global proof for the presence of presynaptic MLT heteroreceptors (i.e. receptor for a transmitter or hormone other than the neuron's own neurotransmitter) capable of regulating neurotransmitter release was still insufficient. A recent protein interaction network analysis established that MT_1 , but not MT_2 , is expressed on presynaptic axon terminal membranes in the hypothalamus, striatum, cortex, and hippocampus, where it is part of the presynaptic protein network (Benleulmi-Chaachoua *et al.*, 2016). Notably, this study shows a strong physical association between MT_1 and presynaptic proteins such as synapsin, SNAP25, Munc-18 and voltage-gated Cav2.2 channels. Interaction with the latter was responsible for constitutive inhibition of calcium entry by MT_1 in a $G\beta\gamma$ -dependent manner (Benleulmi-

Chaachoua *et al.*, 2016).

These recent findings provide strong support for the involvement of MTRs in synaptic functions, particularly in neurotransmitter release as indicated by previous studies. Indeed, activation of MTRs has been implicated in the inhibition of ^3H -dopamine release from the ventral hippocampus, medulla pons, preoptic area and hypothalamus (median and posterior) (Dubocovich, 1983; Zisapel *et al.*, 1982). This effect followed a diurnal rhythm in the hypothalamus with a maximum and a minimum observed at ZT 5 and ZT 13-15, respectively (Zisapel *et al.*, 1985). 6-Chloromelatonin-mediated modulation of norepinephrine turnover via activation of presynaptic MLT heteroreceptors was demonstrated in hypothalamus (Fang *et al.*, 1990). In this model, luzindole, applied during the night when MLT levels are high, accelerated norepinephrine turnover suggesting the involvement of MTRs stimulated by endogenous MLT (Fang *et al.*, 1990). The presence of presynaptic MLT heteroreceptors on retino-hypothalamic fibers innervating superficial retinorecipient layers of the avian optic tectum has been inferred by the presence of 2- ^{125}I -IMLT binding sites and its decrease following transection of the retinotectal pathway (Krause *et al.*, 1992; Krause *et al.*, 1994). The function of these presynaptic MTR is currently unknown but a modulatory role of light input to visual and circadian target responses is likely. Recent electrophysiological evidence suggest that MLT acting through presynaptic MTRs increases glutamatergic neurotransmission in the habenula, an effect blocked by luzindole (Evely *et al.*, 2016). Finally, it is worth mentioning in this context that MTRs have been first shown to be involved in the inhibition of depolarization-evoked calcium-dependent neurotransmitter (dopamine) release from amacrine cells in the chick and rabbit retina (Dubocovich, 1985; Dubocovich, 1983). These mammalian functional presynaptic heteroreceptors were used to establish the first structure-activity-relationship for MTR ligands, which correlated with the pharmacological profile of MT_2 (Dubocovich *et al.*, 1997), and to identify and

pharmacologically characterize the first competitive MTR ligands, luzindole and 4P-PDOT (Dubocovich, 1988).

In summary, proteomic studies of the MT₁ interactome revived interest in the function of presynaptic MTRs and reinforced previous functional studies indicating the role of presynaptic MTRs in neurotransmitter release. Use of MTR KO mouse models will be particularly instrumental in this context, as they will clarify the respective roles of MT₁ and MT₂. Based on current data, a predominant role of MT₁ in presynaptic functions, like neurotransmitter release, and a potential role of MT₂ in axogenesis and synapse formation can be postulated.

Melatonin receptors in neurodegenerative diseases. Altered expression of MTRs has been frequently reported in neurodegenerative diseases and psychiatric disorders, including Alzheimer's disease (AD), Parkinson disease (PD), Huntington disease (HD) and ASD. In AD patients, MT₁ expression in the SCN and MT₂ expression in the hippocampus are reduced compared to control subjects in *post-mortem* brains (Wu *et al.*, 2007). Intriguingly, higher expression of MT₁ was detected in hippocampal arteries of AD brains (Savaskan *et al.*, 2002), which might be due to a compensatory response to the low levels of circulating MLT in these patients (Zhou *et al.*, 2003). These observations suggest that the expression of MTRs under pathological conditions can be differentially regulated depending on the brain area. In PD patients, down-regulation of MT₁ and MT₂ expression was observed in the substantia nigra and amygdala, the two most relevant areas in PD pathogenesis (Adi *et al.*, 2010). Small case-control studies accessing MT₁ and MT₂ expressions in HD patients showed no changes in the SCN (van Wamelen *et al.*, 2013), while decreased expression of MT₁, but not of MT₂, was detected in the striatum (Wang *et al.*, 2011). Interestingly, the progressive loss of MT₁ correlates with HD severity, also confirmed in a mice model of HD (Wang *et al.*, 2011). In

ASD patients no information on MT₁ and MT₂ expression is available but several MT₁ and MT₂ mutants with strongly reduced function have been identified (Chaste *et al.*, 2010).

Additional evidence supports the emerging concept of MTR dysfunction as a permissive condition favoring the development and/or progression of neurodegenerative diseases. The neuroprotective effect of endogenous and exogenous MLT has been demonstrated in different systems (reviewed by (Escribano *et al.*, 2014)). In a neuroinflammatory model induced by LPS administration, cerebellar neuronal death was observed only in animals pre-treated with luzindole (Pinato *et al.*, 2015). Similarly, depletion of endogenous MLT by pinealectomy caused spontaneous neuronal loss in the hippocampal CA1 area, which was prevented by treatment with agomelatine (Tchekalarova *et al.*, 2016). The requirement of MTRs for the neuroprotective action of MLT has also been elegantly demonstrated in a series of *in vitro* studies in which luzindole treatment or siRNA-mediated knockdown of MT₁ enhanced neuronal vulnerability to cell death (Wang *et al.*, 2011). Different cell stressor conditions such as temperature shift or treatments with hydrogen peroxide, TNF or with the HD-related protein huntingtin, resulted in reduced levels of MT₁. Accordingly, it has also been shown that the AD-related neurotoxic amyloid beta peptide (A β) impairs the function of MTRs (Cecon *et al.*, 2015), implying that MTRs and MLT signaling are among the primary molecular targets affected in the course of AD.

Insights in the impact of MTRs on cognitive functions are also obtained from MTR KO mice. MT₂^{-/-} mice show impaired long-term potentiation and performance in memory tests (Larson *et al.*, 2006). However, the double KO MT₁^{-/-}/MT₂^{-/-} mice show no clear differences to WT mice in memory test performances and show increased long-term potentiation responses, even though the deletion of MTRs negatively affected the expression of important proteins for synaptic activity, such as phospho-synapsin and spinophilin (O'Neal-Moffitt *et al.*, 2014). The relevance of MTRs for cognitive performance was

undoubtedly evidenced using an AD mice model lacking MT₁ and MT₂, in which MLT treatment failed to improve mice performance on hippocampal-dependent spatial learning tasks, as observed in the AD mouse model in the presence of MT₁ and MT₂. Impressively, the lack of MTRs *per se* markedly increased the mortality in young AD mice (O'Neal-Moffitt *et al.*, 2015). Finally, the therapeutic use of MLT has been proposed and tested in a number of mice models and clinical trials in several neurodegenerative conditions, including AD (Cardinali *et al.*, 2010; Olcese *et al.*, 2009; Peng *et al.*, 2013; Wade *et al.*, 2014; Zhang *et al.*, 2016), amyotrophic lateral sclerosis (Weishaupt *et al.*, 2006; Zhang *et al.*, 2013), PD (Medeiros *et al.*, 2007; Naskar *et al.*, 2015; Zhang *et al.*, 2016) and HD (van Wamelen *et al.*, 2015). The therapeutic use of MLT is usually associated with sleep improvement and better alignment of circadian parameters, and its beneficial effect on neuroprotection and cognitive performance is starting to be recognized (Joshi *et al.*, 2015; Wade *et al.*, 2014). Dysfunction or down-regulation of MTRs is likely to be part of the primary pathophysiological mechanisms rather than a consequence of advanced neurodegeneration and, thus, prophylactic hormonal replacement and/or early-stage intervention strategies to restore MTR expression and function might provide the most efficient result.

Taken together, the subcellular localization and role of MTRs in neuron functions and their participation in neurodegenerative diseases are now starting to be understood and suggest a broad modulatory role of MLT in neuronal function, development and plasticity.

- Melatonin as antioxidant and free radical scavenger

The IUPHAR classifies only clearly identified pharmacological targets in mammals. However, some effects of MLT persist even in the absence of MT₁ and MT₂ or upon complete pharmacological blockage of MTRs indicating the existence of MTR-independent

mechanism, which are still not fully understood. In addition, MTR-dependent and -independent mechanisms can participate simultaneously, as demonstrated by O'Neal-Moffitt *et al.* (2015) regarding the antioxidant and pro-cognitive effects of MLT on AD mice models, for example. Two predominant mechanisms have been put forward to explain MLT's antioxidant and free radical properties: MLT binding to the *MT3* binding site (Dubocovich *et al.*, 2003; Nosjean *et al.*, 2000) and to the cytosolic enzyme quinone reductase 2 (QR2) (Dubocovich *et al.*, 2003; Nosjean *et al.*, 2000), and MLT scavenging of free radicals, which has been suggested to be an electron donor (for a review see (Tan *et al.*, 2015). Binding of MLT to intracellular targets is readily achieved due to the hydrophilic nature of this indolamine. MLT binds with nanomolar affinity to *MT3*/*QR2* binding sites but shows a pharmacological profile distinct from *MT1* and *MT2*. The order of affinities for the *MT3* binding site is 2-iodomelatonin > N-acetyl-serotonin > MLT (Dubocovich, 1995; Nosjean *et al.*, 2000), the one for *MT1* and *MT2* is 2-iodomelatonin > MLT >>>> N-acetyl-serotonin. MCA-NAT (5-methoxycarbonylamino-N-acetyltryptamine), prazosin and N-acetyltryptamine are selective ligands for the membrane *MT3* binding site (Dubocovich, 1995; Molinari *et al.*, 1996; Nosjean *et al.*, 2000). Nosjean *et al.* (2000) showed that a cytosolic binding site identified as QR2 has the pharmacological characteristics of the membrane *MT3* binding site. QR2 is a cytosolic flavin adenine dinucleotide (FAD)-dependent flavoprotein that reduces menadione and other quinones by using *N*-ribosyl- and *N*-alkyldihydronicotinamides as the co-substrates (Liao *et al.*, 1961) thus acting as a detoxifying enzyme to increase the antioxidant defense (Jockers *et al.*, 2008). There are still open questions as to whether the MLT binding site on QR2 corresponds to the *MT3* binding site, in particular regarding those sites that are membrane associated.

Several physiological effects of MLT such as inhibition of leukocytes adhesion to rat endothelial cell layer were reported to be mimic by *MT3* agonists (Lotufo *et al.*, 2001). Similar observations were made for the expression of adhesion molecules by granulocytes (Cernysiov *et al.*, 2015), the increase in dopamine levels in chick retina (Sampaio Lde *et al.*, 2014) and the reduction of intraocular pressure (IOP) in rabbits (Alarma-Estrany *et al.*, 2009). However, it has been questioned whether the functional effects of MCA-NAT are indeed mediated by QR2, as the lack of QR2 did not prevent the MCA-NAT-induced reduction on IOP, and overexpression of QR2 did not promote receptor-like responses (Vincent *et al.*, 2010). In addition, MCA-NAT turned out to be a partial agonist for *MT*₁ and *MT*₂ at submicromolar concentrations suggesting the possibility that some of the MCA-NAT effects might be mediated by *MT*₁ and/or *MT*₂ (Vincent *et al.*, 2010).

MLT and metabolites with or without open ring structures have been described to be potent electron donors. Cyclic-3-hydroxymelatonin, N1-acetyl-5-methoxykynuramine (secondary metabolite) (AMK, tertiary metabolite), and N-acetyl-N-formyl-5-methoxykynuramine (AFMK, quaternary metabolite) scavenge free radicals neutralizing reactive oxygen and nitrogen species (Ressmeyer *et al.*, 2003; Tan *et al.*, 2007; Zavala-Oseguera *et al.*, 2014). Hence one MLT molecule and its associated metabolites are believed to scavenge a large number of reactive species and thus the overall antioxidant capacity of MLT is believed to be superior of other well known antioxidants (e.g. vitamin C, vitamin E, etc.,) under *in vitro* or *in vivo* conditions (Gitto *et al.*, 2001; Ortiz *et al.*, 2013; Sharma *et al.*, 2006). However, the ability of MLT in reducing oxidative stress does not only rely on donating electrons. Indeed, by acting on *MT*₁ and *MT*₂, low pM and low nM concentrations of MLT increase the expression or activity of enzymes such as superoxide dismutase, catalase and glutathione peroxidase, which are involved in oxygen detoxification (Rosen *et al.*, 2009).

Thus, depending on the dose of exogenous or endogenous MLT, receptor-dependent or -independent mechanisms may be involved. A further twist in the interplay between receptor-dependent and -independent processes could arise from the fact that MLT by changing the redox state of the cell might influence receptor mediated functions. Indeed the function of several GPCRs has been shown to be sensitive to the cellular redox state. Whether this is also the case for MTRs, has to be addressed in future studies. Although endogenous MLT levels are typically considered to range from low pM to low nM concentrations much higher concentrations may be reached locally in the brain (Legros *et al.*, 2014) and in activated immune cells (Conti *et al.*, 2000). In addition, melatonin can be actively taken up through the GLUT1 glucose transporter (Hevia *et al.*, 2015). In conclusion, the role of the *MT3* binding site is still not fully understood and warrants further attention. Concerning the free radical scavenging properties of MLT, it is surprising that still opposing opinions are in the literature. Overall, the antioxidant effects of MLT appear to be complex, relying on a mixture of MTR-dependent and -independent processes.

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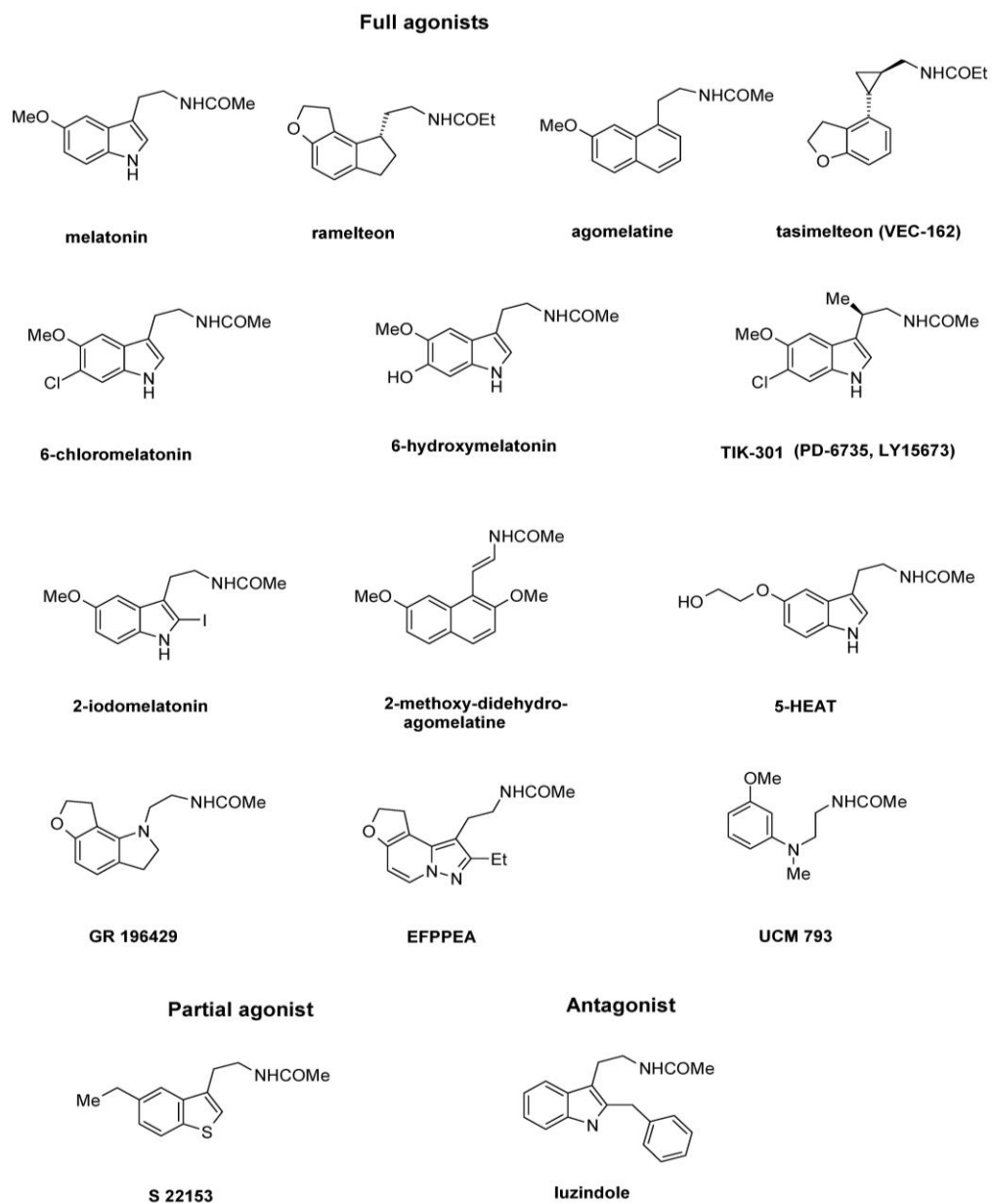


Figure 1. Structures of nonselective MT₁/MT₂ ligands. 5-HEAT, 5-hydroxyethoxy-N-acetyltryptamine; EFPPEA, ethyl-furo-pyrazolo-pyridine-ethyl-acetamide.

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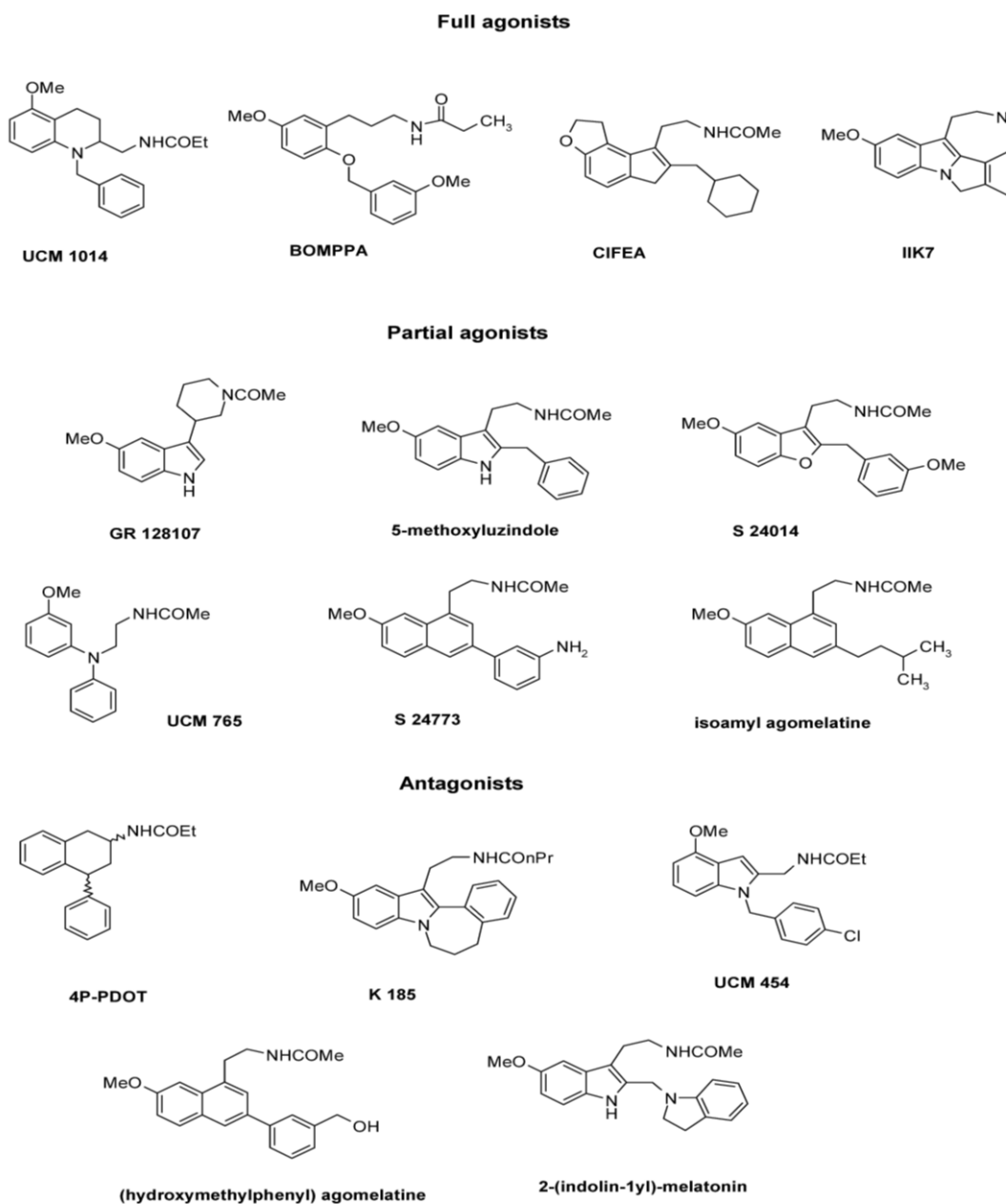


Figure 2. Structures of MT2-selective ligands. BOMPPA, benzyloxy-methoxyphenyl-propylamide; CIFEA, cyclohexylmethyl-indenofurane-ethylacetamide; 4P-PDOT, 4-phenyl-2-propionamidotetralin.

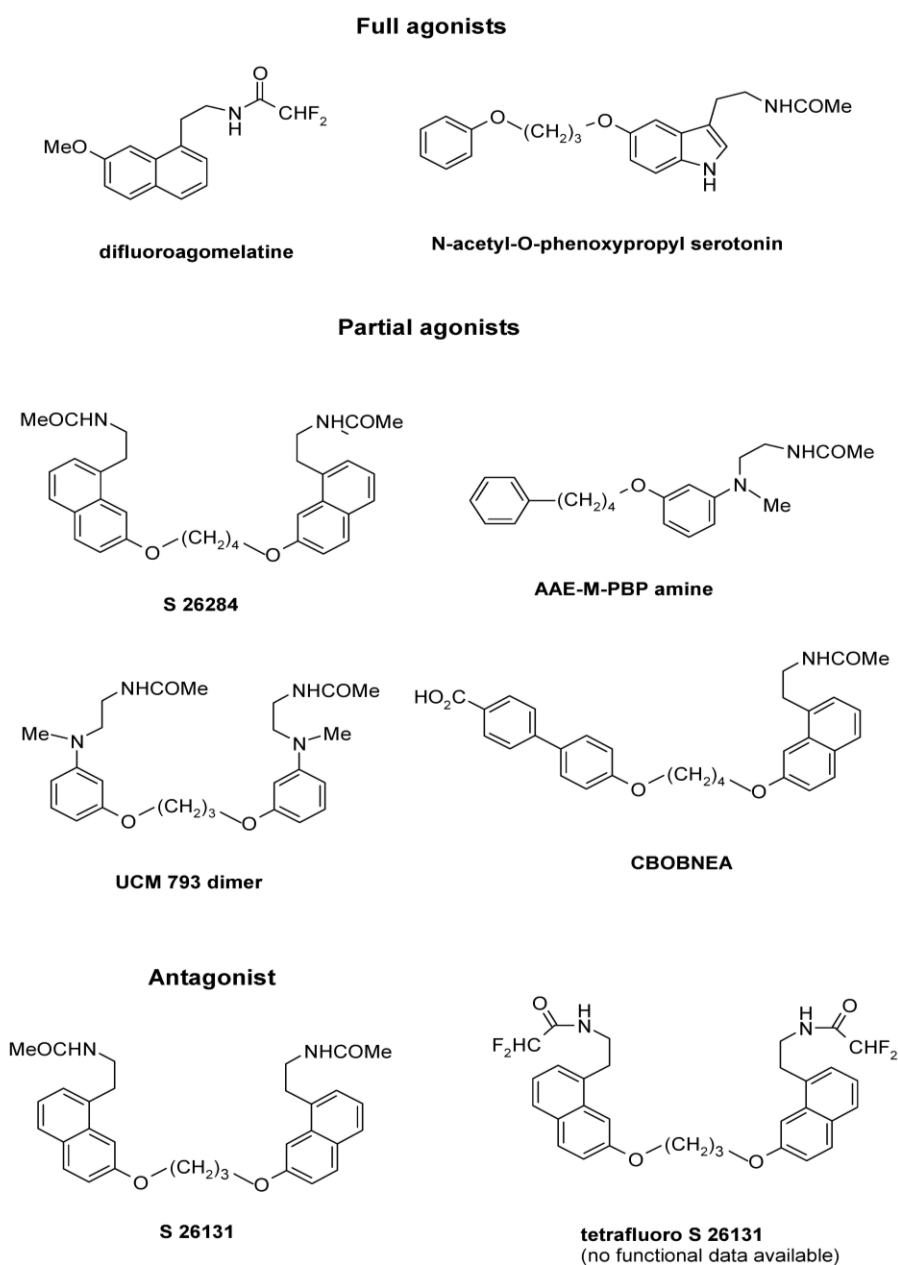


Figure 3. Structures of MT₁-selective ligands. CBOBNEA, carboxybiphenyloxy-butoxy-naphthalene-ethylacetamide; AAE M PBP amine, acetylaminethyl-methyl-phenylbutoxyphenyl- amine.

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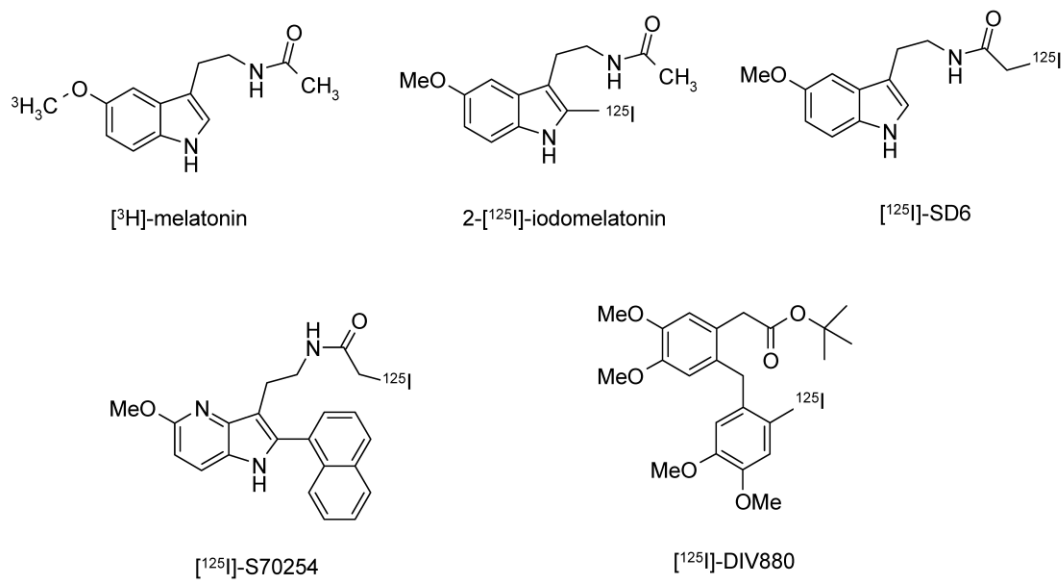


Figure 4. Structures of radioligands used to determine binding affinity for MT₁ and MT₂.

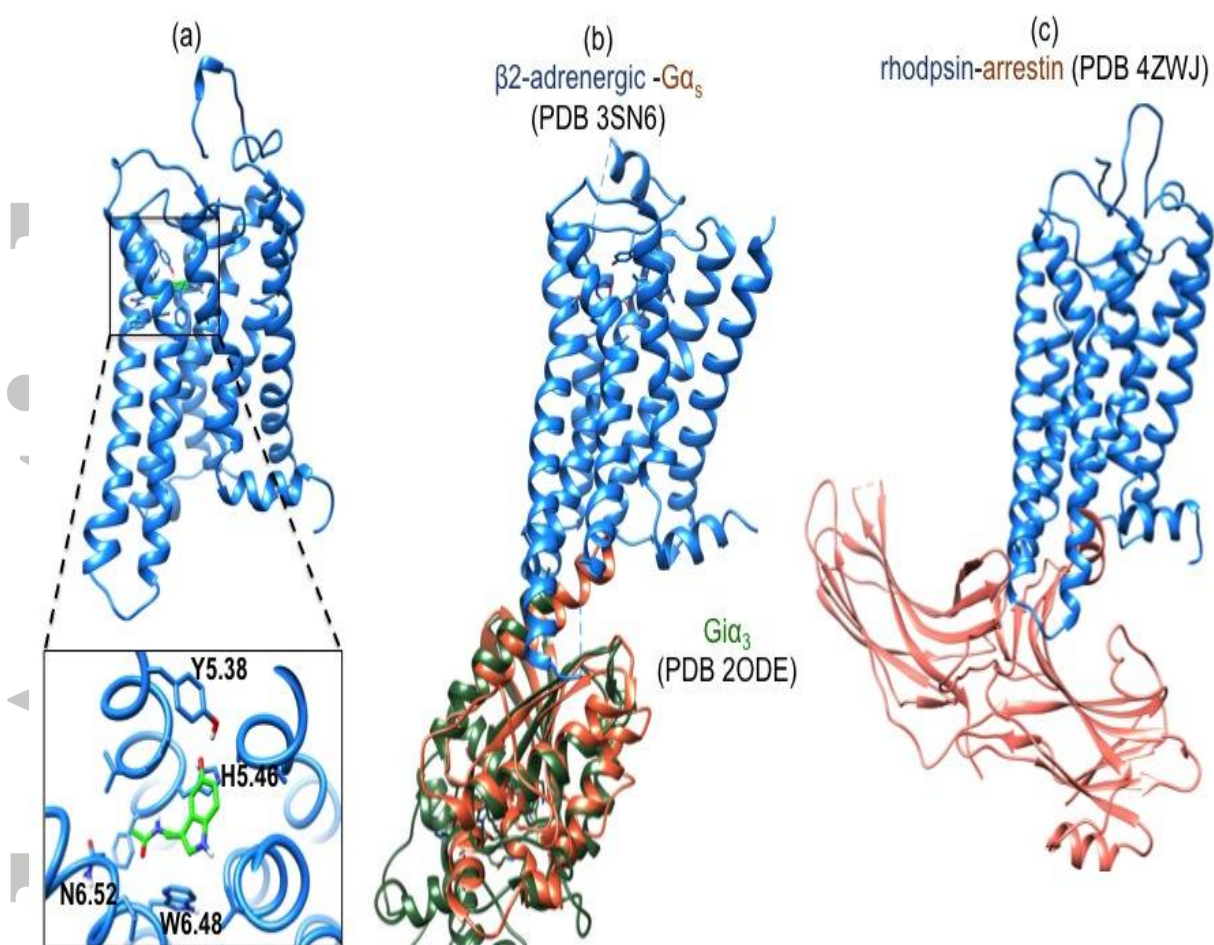


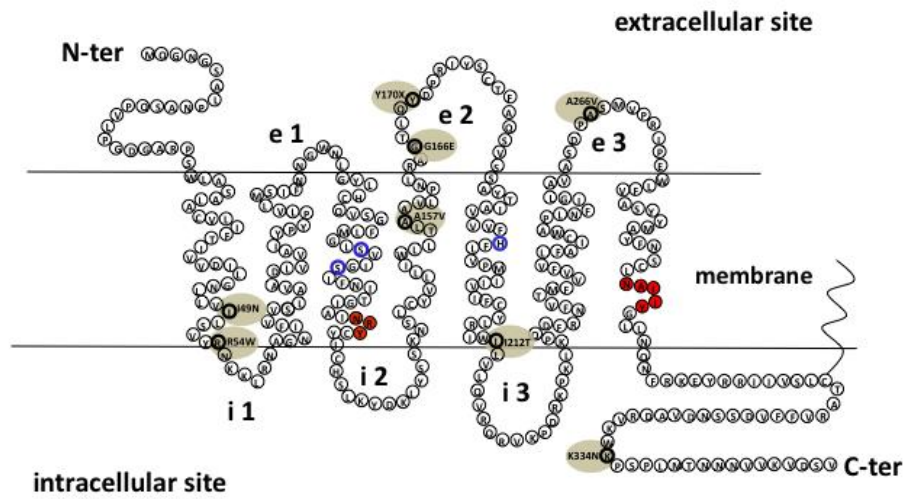
Figure 5. Structures useful for the study of MT₁ receptor structure-function relationships.

MT₁*-MLT (a) was derived from active forms of rhodopsin, β₂-adrenergic and A_{2A} adenosine receptors (unpublished data, N.R.). Docking of melatonin in the solvent-accessible cavity was achieved by energy relaxation by 300 ns molecular dynamics simulations. The structure of the MT₁*-MLT-Giα₃ complex could be modeled on the basis of the sequence homology with the β₂-adrenoceptor-Gα_s structure (Rasmussen *et al.*, 2011b), and the homology between Gα_s and Giα₃ (Soundararajan *et al.*, 2008) (b) whereas the structure of the MT₁*-MLT-arrestin complex could be modeled based on the crystalized rhodopsin-arrestin complex (Kang *et al.*, 2015) (c).

Accepted

a)

MT₁



b)

MT₂

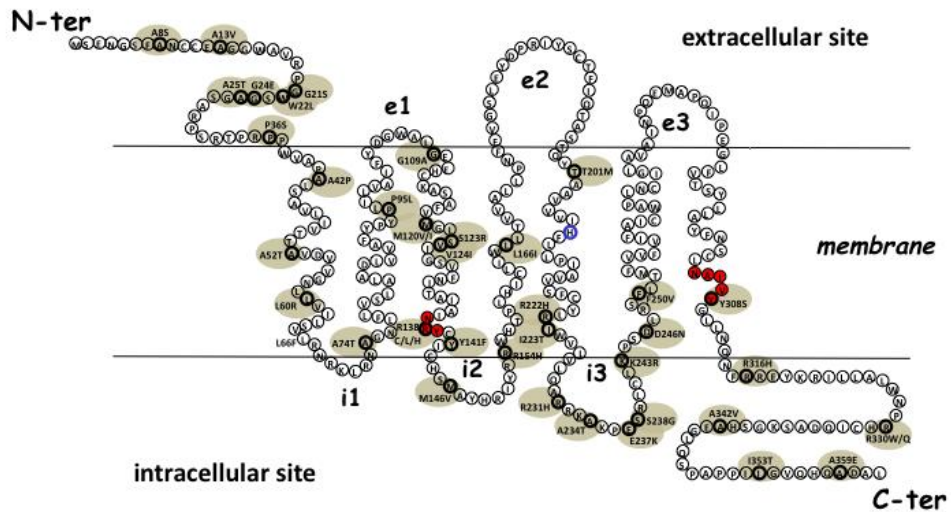


Figure 6: Distribution of non-synonymous MT₁ (a) and MT₂ (b) variants identified in various human populations. Position of variants are highlighted in light brown. Typical signatures of MTRs like the ^{3.49}NR^{3.51} motif and the ^{7.49}NAXXY^{7.53} motif are highlighted in red. Residues suspected to be directly involved in MLT binding (S3.35 and S3.39 in MT₁ and H5.46 in both MT₁ and MT₂) are highlighted with a blue circle. The putative palmitoylation site at C314 is indicated in MT₁.

Table I: Summary of reported MT receptor homology models.

Homology models	Crystal templates	Binding amino acids	Reference
MT ₁ -ramelteon (non-selective agonist)	Inactive rhodopsin (Palczewski et al., 2000)	Y175(E2), S182(E2), V5.43, H5.46	Uchikawa et al., 2002 ²
MT ₁ -agomelatine (non-selective agonist)	Inactive rhodopsin (Okada et al., 2002)	L2.46, M3.32, S3.35 , S3.39 , H5.46 , F5.47, P5.50	Voronkov et al., 2005
MT ₁ -melatonin (non-selective agonist)	Inactive rhodopsin (Okada et al., 2002)	L2.46, M3.32, S3.35 , V3.36, S3.39 , H5.46 , F5.47, F6.44, W6.48, L6.51, N6.52, N7.49	Farce et al., 2008
MT ₁ -2phenylmelatonin (selective agonist)	Active β 2- adrenergic (Rasmussen et al., 2011)	M3.32, G3.33, Y5.38, H5.46 , W6.48, L6.51, N6.52, Y7.39, A7.42, Y7.43	Rivara et al., 2012
MT ₂ -2iodomelatonin (non-selective agonist)	Inactive rhodopsin (Okada et al., 2002)	V5.42 , H5.46 , N6.52, L6.56, Y7.43	Mazna et al., 2004
MT ₂ -UCM454 (selective antagonist)	Inactive rhodopsin (Okada et al., 2004)	V3.36, I3.37, V3.40, Y183(E2), H5.46 , F5.47, P5.50, I5.51, F6.44, W6.48	Rivara et al., 2005
MT ₂ -melatonin	Inactive rhodopsin (Okada et al., 2004)	L2.46, A2.49, S3.35, I3.37, S3.39, V5.42 , V5.43, H5.46 , F5.47	Voronkov et al., 2005
MT ₂ -melatonin	Inactive rhodopsin (Okada et al., 2002)	S3.35, V3.36, S3.39, V5.42 , H5.46 , W6.48, Y7.43	Chugunov et al., 2006
MT ₂ -melatonin	Inactive rhodopsin (Okada et al., 2002)	G3.33, V3.36, I3.37, N4.60 , L4.57, T191(E2), Y5.38, H5.46	Farce et al., 2008
MT ₂ -melatonin	Active rhodopsin (Scheerer et al., 2008)	A3.29, V3.36, N4.60 , H5.46 , W6.48, L6.51	Zefirova et al. 2011
MT ₂ -acetylaminethyl tetralin (selective partial agonist)	Active β 2- adrenergic (Rasmussen et al., 2011)	M3.32, V3.36, N4.60 , H5.46 , W6.48, N6.52, Y7.43	Pala et al., 2013

According to Ballesteros numbering, amino acids critical for ligand binding based on site-directed mutagenesis data (Conway et al., 2001; Gerdin et al., 2003; Kokkola et al., 2003) are displayed in italic and bold.

Table II: Biologically Important MT₁ Variants.

Common (minor allelic frequency (MAF) >1%), rare (MAF 0.1%–1%) variants.

Amino acid change	Type of variant	Description	References
I49N	Missense mutation	Rare variant identified in autism spectrum disorder patients, impaired cell surface expression, melatonin binding, cAMP inhibition and ERK1/1 activation	Chaste et al., 2010
R54W	Missense mutation	Common variant identified in control population without obvious functional defect	Ebisawa et al., 1999
A157V	Missense mutation	Common variant identified in control population without obvious functional defect	Chaste et al., 2010; Ebisawa et al., 1999
G166E	Missense mutation	Common variant identified in control population, impaired cell surface expression, reduced cAMP inhibition and ERK1/2 activation	Chaste et al., 2010
Y170X	Nonsense mutation	Rare variant identified in attention-deficit hyperactivity disorder (ADHD) patient, premature STOP codon with impaired cell surface expression and cAMP inhibition	Chaste et al., 2011
I212T	Missense mutation	Common variant identified in control population, impaired cell surface expression, cAMP inhibition and reduced ERK1/2 activation	Chaste et al., 2010
A266V	Missense mutation	Common variant identified in control population with reduced ERK1/2 activation	Chaste et al., 2010
K334N	Missense mutation	Rare variant identified in control population with reduced cAMP inhibition	Chaste et al., 2010

Table III: Biologically Important MT₂ Variants. Common (minor allelic frequency (MAF) >1%), rare (MAF 0.1%–1%) and very rare (MAF < 0.1%) variants

Amino acid change	Type of variant	Description	References
A8S	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012
A13V	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012
G21S	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012
W22L	Missense mutation	Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, impaired G _i protein activation	Bonnefond et al., 2012
G24E	Missense mutation	Common variant, not associated with type 2 diabetes risk but associated with prevalence of obesity and increased BMI shown in one study but not in another	Andersson et al., 2010; Bonnefond et al., 2012; Chaste et al., 2010; Ebisawa et al., 2000
A25T	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012
P36S	Missense mutation	Very rare variant identified in type 2 diabetes patients, without obvious functional defect	Bonnefond et al., 2012
A52T	Missense mutation	Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, impaired G _i protein activation	Bonnefond et al., 2012
L66F	Missense mutation	Very rare variant identified in control population without obvious functional defect	Ebisawa et al., 2000
A74T	Missense mutation	Very rare variant identified in control population and type 2 diabetes patients, associated with type 2 diabetes risk, impaired G _i protein activation	Bonnefond et al., 2012

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Amino acid change	Type of variant	Description	References
G109A	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012
M120V	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012
M120I	Missense mutation	Very rare variant identified in population with impaired fasting glucose and control population without obvious functional defect	Bonnefond et al., 2012
S123R	Missense mutation	Very rare variant identified in population with impaired fasting glucose and control population without obvious functional defect	Bonnefond et al., 2012
V124I	Missense mutation	Very rare variant identified in several populations including type 2 diabetes and ASD without obvious functional defect in one study and impaired ERK1/2 activation in another	Andersson et al., 2010; Bonnefond et al., 2012; Chaste et al., 2010
R138C	Missense mutation	Rare variant, not associated with type 2 diabetes risk, no Gi and ERK1/2 activation	Andersson et al., 2010; Bonnefond et al., 2012; Chaste et al., 2010
R138L	Missense mutation	Very rare variant identified in control population, associated with type 2 diabetes risk, impaired G _i protein activation	Bonnefond et al., 2012
R138H	Missense mutation	Very rare variant identified in control population, associated with type 2 diabetes risk, impaired Gi protein activation	Bonnefond et al., 2012
Y141F	Missense mutation	Very rare variant identified in type 2 diabetes patients, without obvious functional defect	Bonnefond et al., 2012

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Amino acid change	Type of variant	Description	References
M146V	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012
R154H	Missense mutation	Very rare variant identified in control population and type 2 diabetes patients without obvious functional defect	Bonnefond et al., 2012
L166I	Missense mutation	Very rare variant identified in control population, associated with type 2 diabetes risk, impaired G _i protein activation	Bonnefond et al., 2012
T201M	Missense mutation	Very rare variant identified in type 2 diabetes patients, without obvious functional defect	Bonnefond et al., 2012
R222H	Missense mutation	Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, impaired G _i protein activation	Bonnefond et al., 2012
I223T	Missense mutation	Very rare variant identified in type 2 diabetes patients, without obvious functional defect	Bonnefond et al., 2012
R231H	Missense mutation	Rare variant, not associated with type 2 diabetes risk	Andersson et al., 2010; Bonnefond et al., 2012; Chaste et al., 2010
A234T	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012
E237K	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012

Amino acid change	Type of variant	Description	References
S238G	Missense mutation	Very rare variant identified in type 2 diabetes patients, without obvious functional defect	Bonnefond et al., 2012
K243R	Missense mutation	Common variant, not associated with type 2 diabetes risk	Bonnefond et al., 2012
D246N	Missense mutation	Very rare variant identified in type 2 diabetes patients, without obvious functional defect	Bonnefond et al., 2012
F250V	Missense mutation	Very rare variant identified in type 2 diabetes patients with impaired ERK1/2 activation	Bonnefond et al., 2012
R316H	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012
R330W	Missense mutation	Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, impaired G _i protein activation	Bonnefond et al., 2012
R330Q	Missense mutation	Very rare variant identified in control population without obvious functional defect	Chaste et al., 2010
A342V	Missense mutation	Very rare variant identified in type 2 diabetes patients, without obvious functional defect	Bonnefond et al., 2012
I353T	Missense mutation	Very rare variant identified in type 2 diabetes patients and control population, associated with type 2 diabetes risk, impaired G _i protein activation	Bonnefond et al., 2012
A359E	Missense mutation	Very rare variant identified in control population without obvious functional defect	Bonnefond et al., 2012

Table IV: Mutations in the *MTNR1B* gene associated with susceptibility to type II diabetes.
Very rare (minor allelic frequency (MAF) <0.1%) variants.

Amino acid change	Type of variant	Description	References
A42P	Missense mutation	Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, no melatonin binding and signaling	Bonnefond et al., 2012
L60R	Missense mutation	Very rare variant identified in control population and type 2 diabetes patients, associated with type 2 diabetes risk, no melatonin binding and signaling	Andersson et al., 2010; Bonnefond et al., 2012
P95L	Missense mutation	Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, no melatonin binding and signaling	Bonnefond et al., 2012
Y308S	Missense mutation	Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, no melatonin binding and signaling	Bonnefond et al., 2012

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