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 exit MT\textsubscript{1} and MT\textsubscript{2} encoded by the *MTNRA* and *MTNRB* 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.

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**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 Gᵢₒ-type proteins and MT₁ in addition to G₉-type proteins. In humans, the *MTNRI*A gene encoding MT₁ is located on chromosome 4q35.1 and the *MTNRI*B 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-HT₂c 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 (Kᵢ = 0.03 nM) and hMT₂ (Kᵢ = 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-HT₂C and 5-HT₂B, 
(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). EFPPEA, a high-affinity hMT₁ 
(Kᵢ = 0.062 nM) and hMT₂ (Kᵢ = 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 GTPγS 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 CIFEA (Koike et al., 2011). In imprinting control region (ICR) mice, CIFEA 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 µM concentrations,
which would activate both MT\(_1\) and MT\(_2\), precluding any conclusion about the specific receptor type involved in the regulation of chronobiotic processes. Similarly, doses of the MT\(_2\)-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 MT\(_1\) and MT\(_2\).

Two moderately selective MT\(_2\) 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 MT\(_2\) antagonist 4P-PDOT (Ochoa-Sanchez et al., 2011). In contrast, the non-selective MT\(_1\)-MT\(_2\) agonist UCM793 decreased sleep onset without having an effect on NREM sleep maintenance suggesting that dual MT\(_1\) and MT\(_2\) agonistic activity accounts for the effect on sleep onset, whereas selectivity for MT\(_2\) 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 hMT\(_2\)-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 hMT\(_2\)-selective antagonist with 300- to 1,500-fold higher affinity for hMT\(_2\), is still considered the gold standard for pharmacological characterization of MTRs (Dubocovich et al., 1997). Other MT\(_2\)-selective antagonists, such as K185 (Faust et al., 2000; Sugden et al., 1999), UCM 454 (Rivara et al., 2005), and 2-(indolin-1-yl) melatonin (Zlotos et al., 2009) display ~100-fold higher affinity for hMT\(_2\). 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)₅OPh 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 MT$_2$ subtype are available, discovery of ligands with at least >100-fold selectivity for MT$_1$ remains a challenging task. None of the MT$_1$-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 MT$_1$-MT$_2$ nonselective receptor antagonist luzindole and the MT$_2$-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 $^{125}$I 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-$^{125}$Iiodomelatonin (2-$^{125}$I-MLT), has been extensively used as a high-affinity radioligand for both MT$_1$ and MT$_2$, 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 $[^3]$H-melatonin ($[^3]$H-MLT) established the pharmacological profile of the human recombinant MT$_1$ and MT$_2$, as being identical to that established using 2-$^{125}$I-MLT as a radioligand. However due to the rather low specific activity of this $^3$H-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-1Hindol-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 pKd 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 Kd 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. $^{125}\text{I}}$-SD6 detected as 2-$^{125}\text{I}}$-MLT binding sites in sheep retinal membranes, while the MT$_2$-specific ligands $^{125}\text{I}}$-S70254 and $^{125}\text{I}}$-DIV880 failed to do so.

The MT$_2$-specific $^{125}\text{I}}$-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-$^{125}\text{I}}$-MLT (detecting MT$_1$ and MT$_2$) was observed in several areas but also distinct labeling in others. Absence of labeling by $^{125}\text{I}}$-S70254 in regions that are labeled by 2-$^{125}\text{I}}$-MLT can be explained by low(undetectable) MT$_2$ expression levels. Absence of 2-$^{125}\text{I}}$-MLT labeling in regions labeled by $^{125}\text{I}}$-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 MT$_2$-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 MT$_1$ and MT$_2$ 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 MT$_1$ or/and MT$_2$ 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 MT$_1$ as well as His5.46 in both MT$_1$ and MT$_2$. Although His5.46 seems to be an anchoring residue for polar interactions with the methoxy or amide group of MLT, only a few MT$_1$ 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 3.49NRY3.51 motif instead of the classical 3.49DRY3.51 motif of other rhodopsin-like GPCRs. Another specificity is the replacement of the proline by an alanine residue in the conserved 7.49NPXXY7.53 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-Gi 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 \( \text{MT}_1/\text{MT}_2 \) and \( \text{MT}_1/\text{GPR50} \) 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 \( \text{MT}_1 \) and \( \text{MT}_2 \) to form homo- and heterodimers (Ayoub et al., 2002) with \( \text{MT}_1/\text{MT}_2 \) heterodimers showing a pharmacological profile distinct from \( \text{MT}_2 \) homodimers (Ayoub et al., 2004). Shortly after, \( \text{MT}_1 \) and \( \text{MT}_2 \) were reported to form heterodimers with the orphan GPR50, which completely abolished the function of \( \text{MT}_1 \) in \( \text{MT}_1/\text{GPR50} \) 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 \( \text{MT}_1/\text{MT}_2 \) heterodimers was obtained. In retinal photoreceptor cells, MLT enhances the light sensitivity during the night. The phenotype of \( \text{MT}_1 \) KO (\( \text{MT}_1^{-/-} \)) and \( \text{MT}_2 \) KOs (\( \text{MT}_2^{-/-} \)) mice, the use of type-selective ligands and over-expression of a dominant negative form of \( \text{MT}_2 \) in photoreceptor cells of transgenic mice indicated the exclusive involvement of \( \text{MT}_1/\text{MT}_2 \) 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 MT$_1$ and MT$_2$.

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 MT$_1$ and MT$_2$ 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 MT$_2$/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 MT$_2$/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 MT$_1$ and MT$_2$ 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 *MTNR1A* and *MTNR1B* genes, encoding MT$_1$ and MT$_2$ 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 *MTNR1A* and *MTNR1B* genes in patients with circadian disorders. Two non-synonymous variants were identified in the *MTNR1A* 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 *MTNR1A* 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 *MTNR1A* and ten for *MTNR1B* (Table II, III). The majority of these mutants showed altered receptor function. Particularly deleterious mutants were MT$_1$-I49N, which is devoid of any MLT binding and cell surface expression, and MT$_1$-G166E and MT$_1$-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 (Bonnefond *et al.*, 2012). Whereas only subtle changes in the capacity of G24E and V124I to activate a GoΔ6qi4myr 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) (Bonnefond *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 (Goqi9 chimera and ERK1/2 activation) in 1 rare cases (R138C) and 9 very are 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 \textit{MTNR1B} and T2D (for review see (Karamitri \textit{et al.}, 2013)).

In conclusion, the genetic variability of the \textit{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 \textit{MTNR1A} gene in terms of non-synonymous variants.

\textbf{Melatonin receptor mouse models - update}

\textit{MT}_1^{-/-} mice were created in the late 90s’ followed by the generation of \textit{MT}_2^{-/-} mice in 2003 (Jin \textit{et al.}, 2003; Liu \textit{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 \textit{MT}_1^{-/-} mice, but not in \textit{MT}_2^{-/-} mice, the inhibitory effect of MLT on neuronal activity in the suprachiasmatic nucleus (SCN) is impaired suggesting the involvement of \textit{MT}_1. In contrast, in SCN slices from \textit{MT}_1^{-/-} mice MLT (1-10 pM) phase-shifts the peak of circadian rhythms of neuronal firing by approximately 3 h suggesting the involvement of \textit{MT}_2 (Dubocovich \textit{et al.}, 2005; Liu \textit{et al.}, 1997). Blockade of this effect using the \textit{MT}_2 selective 4P-PDOT antagonist confirmed the latter conclusion shaping a pathway where \textit{MT}_2 phase-shifts the peak of neuronal firing through PLC-PKC signaling pathway (Dubocovich \textit{et al.}, 2005; Hunt \textit{et al.}, 2001) (Mc Arthur \textit{et al.}, 1997). Liu \textit{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 \textit{MT}_1^{-/-} than in WT mice suggesting a role for the \textit{MT}_1 in this response (see detailed discussion in (Dubocovich, 2007)). Together these findings suggest a potential role for both \textit{MT}_1 and \textit{MT}_2 in the phase shift of circadian rhythms of neuronal firing in the SCN slice \textit{in vitro}. The use of \textit{MT}_1^{-/-} mice demonstrated that \textit{MT}_1 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
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 MT$_1$ and MT$_2$ 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; Gianesini 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 (Bonnefond 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 MT$_1$ 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 MT$_2^{-/-}$ mice (Contreras-Alcantara et al., 2010). Furthermore, removal of MT$_1$ or MT$_2$ 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, MT$_1$ 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 MT$_1$ signaling as well (Jilg et al., 2005). MT$_1$ 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 MT$_1$ 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 MT1-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 MT1 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 MT2 and ligands binding to the putative MT3 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) (Cernysov 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 mononuclear cells, including colostral and lineage established cell lineages is blocked by luzindole. Indeed, parasites, bacteria and fungi activate the NF-κB 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 mediated 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-
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 $^3$H-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 transsection 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 MT\textsubscript{1} 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 MT\textsubscript{1} and MT\textsubscript{2}. Based on current data, a predominant role of MT\textsubscript{1} in presynaptic functions, like neurotransmitter release, and a potential role of MT\textsubscript{2} in axogenesis and synapse formation can be postulated.

\textit{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, MT\textsubscript{1} expression in the SCN and MT\textsubscript{2} expression in the hippocampus are reduced compared to control subjects in \textit{post-mortem} brains (Wu \textit{et al.}, 2007). Intriguingly, higher expression of MT\textsubscript{1} was detected in hippocampal arteries of AD brains (Savaskan \textit{et al.}, 2002), which might be due to a compensatory response to the low levels of circulating MLT in these patients (Zhou \textit{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 MT\textsubscript{1} and MT\textsubscript{2} expression was observed in the substantia nigra and amygdala, the two most relevant areas in PD pathogenesis (Adi \textit{et al.}, 2010). Small case-control studies accessing MT\textsubscript{1} and MT\textsubscript{2} expressions in HD patients showed no changes in the SCN (van Wamelen \textit{et al.}, 2013), while decreased expression of MT\textsubscript{1}, but not of MT\textsubscript{2}, was detected in the striatum (Wang \textit{et al.}, 2011). Interestingly, the progressive loss of MT\textsubscript{1} correlates with HD severity, also confirmed in a mice model of HD (Wang \textit{et al.}, 2011). In
ASD patients no information on MT\textsubscript{1} and MT\textsubscript{2} expression is available but several MT\textsubscript{1} and MT\textsubscript{2} mutants with strongly reduced function have been identified (Chaste \textit{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 \textit{et al.}, 2014)). In a neuroinflammatory model induced by LPS administration, cerebellar neuronal death was observed only in animals pre-treated with luzindole (Pinato \textit{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 \textit{et al.}, 2016). The requirement of MTRs for the neuroprotective action of MLT has also been elegantly demonstrated in a series of \textit{in vitro} studies in which luzindole treatment or siRNA-mediated knockdown of MT\textsubscript{1} enhanced neuronal vulnerability to cell death (Wang \textit{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 MT\textsubscript{1}. Accordingly, it has also been shown that the AD-related neurotoxic amyloid beta peptide (\textit{A\textsubscript{\beta}}) impairs the function of MTRs (Cecon \textit{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. MT\textsubscript{2}\textsuperscript{-/-} mice show impaired long-term potentiation and performance in memory tests (Larson \textit{et al.}, 2006). However, the double KO MT\textsubscript{1}\textsuperscript{-/-}/MT\textsubscript{2}\textsuperscript{-/-} 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 \textit{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 (Cardinale 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 MT1 and MT2 at submicromolar concentrations suggesting the possibility that some of the MCA-NAT effects might be mediated by MT1 and/or MT2 (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 MT1 and MT2, 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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All authors contributed equally in the writing and proofreading of the review and RJ
supervised and edited the different contributions.

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MTNR1B G24E variant associates With BMI and fasting plasma glucose in the general


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Figure 1. Structures of nonselective MT$_1$/MT$_2$ ligands. 5-HEAT, 5-hydroxyethoxy-N-acetyltryptamine; EFPPEA, ethyl-furo-pyrazolo-pyridine-ethylacetamide.
Figure 2, Structures of MT2-selective ligands. BOMPPA, benzyloxy-methoxyphenylpropylamide; CIFEA, cyclohexylmethyl-indenofuran-ethylacetamide; 4P-PDOT, 4-phenyl-2-propionamidotetralin.
Figure 3. Structures of MT$_1$-selective ligands. CBOBNEA, carboxybiphenyloxy-butoxy-naphthalene-ethylacetamide; AAE M PBP amine, acetylaminoethyl-methyl-phenylbutoxyphenyl- amine.
Figure 4. Structures of radioligands used to determine binding affinity for MT\textsubscript{1} and MT\textsubscript{2}.
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 A₂A 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-Gi₃ complex could be modeled on the basis of the sequence homology with the β2-adrenoceptor-Gα₅ structure (Rasmussen et al., 2011b), and the homology between Gα₅ and Gi₃ (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).
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 $^{3.49}$NRY$^{3.51}$ 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 1: Summary of reported MT receptor homology models.

<table>
<thead>
<tr>
<th>Homology models</th>
<th>Crystal templates</th>
<th>Binding amino acids</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT&lt;sub&gt;1&lt;/sub&gt;-ramelteon (non-selective agonist)</td>
<td>Inactive rhodopsin</td>
<td>Y175(E2), S182(E2), V5.43, <strong>H5.46</strong></td>
<td>Uchikawa et al., 2002&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>(Palczewski et al., 2000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT&lt;sub&gt;1&lt;/sub&gt;-agomelatine (non-selective agonist)</td>
<td>Inactive rhodopsin</td>
<td>L2.46, M3.32, S3.35, **S3.39, H5.46, F5.47, P5.50</td>
<td>Voronkov et al., 2005</td>
</tr>
<tr>
<td></td>
<td>(Okada et al., 2002)</td>
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<tr>
<td></td>
<td>(Okada et al., 2002)</td>
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<tr>
<td></td>
<td>(Rasmussen et al., 2011)</td>
<td></td>
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</tr>
<tr>
<td>MT&lt;sub&gt;2&lt;/sub&gt;-2iodomelatonin (non-selective agonist)</td>
<td>Inactive rhodopsin</td>
<td>V5.42, **H5.46, N6.52, L6.56, **Y7.43</td>
<td>Mazna et al., 2004</td>
</tr>
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<td></td>
<td>(Okada et al., 2002)</td>
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</tr>
<tr>
<td>MT&lt;sub&gt;2&lt;/sub&gt;-UCM454 (selective antagonist)</td>
<td>Inactive rhodopsin</td>
<td>V3.36, I3.37, V3.40, Y183(E2), **H5.46, F5.47, P5.50, I5.51, F6.44, W6.48</td>
<td>Rivara et al., 2005</td>
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<td>(Okada et al., 2004)</td>
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</tr>
<tr>
<td>MT&lt;sub&gt;2&lt;/sub&gt;-melatonin</td>
<td>Inactive rhodopsin</td>
<td>L2.46, A2.49, S3.35, I3.37, S3.39, **V5.42, V5.43, **H5.46, F5.47</td>
<td>Voronkov et al., 2005</td>
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<td>(Okada et al., 2004)</td>
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<td></td>
</tr>
<tr>
<td>MT&lt;sub&gt;2&lt;/sub&gt;-melatonin</td>
<td>Inactive rhodopsin</td>
<td>S3.35, V3.36, S3.39, **V5.42, H5.46, W6.48, **Y7.43</td>
<td>Chugunov et al., 2006</td>
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<td>(Okada et al., 2002)</td>
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<td></td>
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<tr>
<td>MT&lt;sub&gt;2&lt;/sub&gt;-melatonin</td>
<td>Inactive rhodopsin</td>
<td>G3.33, V3.36, I3.37, **N4.60, L4.57, T191(E2), Y5.38, **H5.46</td>
<td>Farce et al., 2008</td>
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<td></td>
<td>(Okada et al., 2002)</td>
<td></td>
<td></td>
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<tr>
<td>MT&lt;sub&gt;2&lt;/sub&gt;-melatonin</td>
<td>Active rhodopsin</td>
<td>A3.29, V3.36, **N4.60, **H5.46, W6.48, L6.51</td>
<td>Zefirova et al. 2011</td>
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<td></td>
<td>(Scheerer et al., 2008)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT&lt;sub&gt;2&lt;/sub&gt;-acetylamoethyl tetralin (selective</td>
<td>Active β2-adrenergic</td>
<td>M3.32, V3.36, **N4.60, **H5.46, W6.48, N6.52, **Y7.43</td>
<td>Pala et al., 2013</td>
</tr>
<tr>
<td>partial agonist)</td>
<td>(Rasmussen et al., 2011)</td>
<td></td>
<td></td>
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</tbody>
</table>

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>
<thead>
<tr>
<th>Amino acid change</th>
<th>Type of variant</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>I49N</td>
<td>Missense mutation</td>
<td>Rare variant identified in autism spectrum disorder patients, impaired cell surface expression, melatonin binding, cAMP inhibition and ERK1/2 activation</td>
<td>Chaste et al., 2010</td>
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<tr>
<td>R54W</td>
<td>Missense mutation</td>
<td>Common variant identified in control population without obvious functional defect</td>
<td>Ebisawa et al., 1999</td>
</tr>
<tr>
<td>A157V</td>
<td>Missense mutation</td>
<td>Common variant identified in control population without obvious functional defect</td>
<td>Chaste et al., 2010; Ebisawa et al., 1999</td>
</tr>
<tr>
<td>G166E</td>
<td>Missense mutation</td>
<td>Common variant identified in control population, impaired cell surface expression, reduced cAMP inhibition and ERK1/2 activation</td>
<td>Chaste et al., 2010</td>
</tr>
<tr>
<td>Y170X</td>
<td>Nonsense mutation</td>
<td>Rare variant identified in attention-deficit hyperactivity disorder (ADHD) patient, premature STOP codon with impaired cell surface expression and cAMP inhibition</td>
<td>Chaste et al., 2011</td>
</tr>
<tr>
<td>I212T</td>
<td>Missense mutation</td>
<td>Common variant identified in control population, impaired cell surface expression, cAMP inhibition and reduced ERK1/2 activation</td>
<td>Chaste et al., 2010</td>
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<tr>
<td>A266V</td>
<td>Missense mutation</td>
<td>Common variant identified in control population with reduced ERK1/2 activation</td>
<td>Chaste et al., 2010</td>
</tr>
<tr>
<td>K334N</td>
<td>Missense mutation</td>
<td>Rare variant identified in control population with reduced cAMP inhibition</td>
<td>Chaste et al., 2010</td>
</tr>
<tr>
<td>Amino acid change</td>
<td>Type of variant</td>
<td>Description</td>
<td>References</td>
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<tr>
<td>A8S</td>
<td>Missense mutation</td>
<td>Very rare variant identified in control population without obvious functional defect</td>
<td>Bonnefond et al., 2012</td>
</tr>
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<td>A13V</td>
<td>Missense mutation</td>
<td>Very rare variant identified in control population without obvious functional defect</td>
<td>Bonnefond et al., 2012</td>
</tr>
<tr>
<td>G21S</td>
<td>Missense mutation</td>
<td>Very rare variant identified in control population without obvious functional defect</td>
<td>Bonnefond et al., 2012</td>
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<tr>
<td>W22L</td>
<td>Missense mutation</td>
<td>Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, impaired Gi protein activation</td>
<td>Bonnefond et al., 2012</td>
</tr>
<tr>
<td>G24E</td>
<td>Missense mutation</td>
<td>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</td>
<td>Andersson et al., 2010; Bonnefond et al., 2012; Chaste et al., 2010; Ebisawa et al., 2000</td>
</tr>
<tr>
<td>A25T</td>
<td>Missense mutation</td>
<td>Very rare variant identified in control population without obvious functional defect</td>
<td>Bonnefond et al., 2012</td>
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<tr>
<td>P36S</td>
<td>Missense mutation</td>
<td>Very rare variant identified in type 2 diabetes patients, without obvious functional defect</td>
<td>Bonnefond et al., 2012</td>
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<tr>
<td>A52T</td>
<td>Missense mutation</td>
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<td>L66F</td>
<td>Missense mutation</td>
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<td>A74T</td>
<td>Missense mutation</td>
<td>Very rare variant identified in control population and type 2 diabetes patients, associated with type 2 diabetes risk, impaired Gi protein activation</td>
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<tr>
<td>Amino acid change</td>
<td>Type of variant</td>
<td>Description</td>
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<td>M120I</td>
<td>Missense mutation</td>
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<td>S123R</td>
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<tr>
<td>V124I</td>
<td>Missense mutation</td>
<td>Very rare variant identified in several populations including type 2 diabetes and ADSD without obvious functional defect in one study and impaired ERK1/2 activation in another</td>
<td>Andersson et al., 2010; Bonnefond et al., 2012; Chaste et al., 2010</td>
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<tr>
<td>R138C</td>
<td>Missense mutation</td>
<td>Rare variant, not associated with type 2 diabetes risk, no Gi and ERK1/2 activation</td>
<td>Andersson et al., 2010; Bonnefond et al., 2012; Chaste et al., 2010</td>
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<tr>
<td>R138L</td>
<td>Missense mutation</td>
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<td>R138H</td>
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<td>Y141F</td>
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<td>Amino acid change</td>
<td>Type of variant</td>
<td>Description</td>
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<td>R154H</td>
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<td>L166I</td>
<td>Missense mutation</td>
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<td>Bonnefond et al., 2012</td>
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<td>R222H</td>
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<td>Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, impaired $G_i$ protein activation</td>
<td>Bonnefond et al., 2012</td>
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<td>I223T</td>
<td>Missense mutation</td>
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<td>Missense mutation</td>
<td>Rare variant, not associated with type 2 diabetes risk</td>
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<td>Missense mutation</td>
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<td>Bonnefond et al., 2012</td>
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<td>E237K</td>
<td>Missense mutation</td>
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<td>A359E</td>
<td>Missense mutation</td>
<td>Very rare variant identified in control population without obvious functional defect</td>
<td>Bonnefond et al., 2012</td>
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</table>
Table IV: Mutations in the *MTNR1B* gene associated with susceptibility to type II diabetes. Very rare (minor allelic frequency (MAF) <0.1%) variants.

<table>
<thead>
<tr>
<th>Amino acid change</th>
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<th>Description</th>
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<td>A42P</td>
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<td>Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, no melatonin binding and signaling</td>
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<td>L60R</td>
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<td>Andersson et al., 2010; Bonnefond et al., 2012</td>
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<td>P95L</td>
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<tr>
<td>Y308S</td>
<td>Missense mutation</td>
<td>Very rare variant identified in type 2 diabetes patients, associated with type 2 diabetes risk, no melatonin binding and signaling</td>
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