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Themed Section: Recent Developments in Research of Melatonin and its Potential Therapeutic Applications

REVIEW ARTICLE

Melatonin receptors: molecular pharmacology and signalling in the context of system bias

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Melatonin, *N*-acetyl-5-methoxytryptamine, an evolutionally old molecule, is produced by the pineal gland in vertebrates, and it binds with high affinity to melatonin receptors, which are members of the GPCR family. Among the multiple effects attributed to melatonin, we will focus here on those that are dependent on the activation of the two mammalian MT_1 and MT_2 melatonin receptors. We briefly summarize the latest developments on synthetic melatonin receptor ligands, including multi-target-directed ligands, and the characterization of signalling-biased ligands. We discuss signalling pathways activated by melatonin receptors that appear to be highly cell- and tissue-dependent, emphasizing the impact of system bias on the functional outcome. Different proteins have been demonstrated to interact with melatonin receptors, and thus, we postulate that part of this system bias has its molecular basis in differences of the expression of receptor-associated proteins including heterodimerization partners. Finally, bias at the level of the receptor, by the expression of genetic receptor variants, will be discussed to show how a modified receptor function can have an effect on the risk for common diseases like type 2 diabetes in humans.

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Abbreviations

4P-PDOT, 4-phenyl-2-propionamidotetralin; AD, Alzheimer's disease; ASD, autism spectrum disorder; miRNA, microRNA; MUPP1, multi-PDZ domain protein 1; PGC-1α, PPAR-γ coactivator; RGS, regulator of G-protein signalling; SCN, suprachiasmatic nucleus; SIRT, sirtuin histone deacetylase; T2D, type 2 diabetes

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Introduction

The **melatonin receptor** family is one of the GPCR subfamilies (Jockers *et al.*, 2016). In higher vertebrates, the melatonin receptor family is composed of two members, **MT_1** and **MT_2** (Reppert *et al.*, 1994; 1995a), which have high affinity for the natural ligand **melatonin**. Human MT_1 and MT_2 receptors are composed of 350 and 362 amino acids and show an overall sequence homology of 55% and 70% in the transmembrane domain (Oishi and Jockers, 2016). Based on the high-sequence homology (50%) with MT_1 and MT_2, the melatonin-related receptor, also called **GPR50**, was classified as another member of the melatonin receptor family (Reppert *et al.*, 1996a). However, GPR50 does not bind to melatonin or any other known ligand and, thus, is still considered an orphan receptor (Reppert *et al.*, 1996a; Ngo *et al.*, 2016). Interestingly, GPR50 has been proposed to be the mammalian orthologue of Mel1c, a high-affinity melatonin receptor found in non-mammalian vertebrates (Dufourny *et al.*, 2008). The main focus of this review will be on the function of MT_1 and MT_2 receptors, whereas GPR50 will be considered as a regulatory protein that associates with melatonin receptors.

The natural ligand of MT_1 and MT_2 receptors is melatonin. Melatonin is an evolutionally very old molecule, which is synthesized in many organisms such as bacteria, protists, fungi, macroalgae, plants and animals. In vertebrates, melatonin is the main hormone produced by the pineal gland and follows a circadian pattern, synchronized to the dark phase of the environmental light/dark cycle. In humans, MT_1 and MT_2 receptors are expressed in brain and several peripheral organs (Table 1). Because both receptors are mainly coupled to $G\alpha_{i/o}$ proteins, decreased intracellular levels of the second messenger cAMP is the most commonly reported signalling pathway triggered by melatonin. Melatonin receptors regulate circadian rhythms, sleep, seasonal reproduction, immune functions, retinal physiology and glucose homeostasis (Dubocovich *et al.*, 2010; Jockers *et al.*, 2016). The establishment of MT_1 (Liu *et al.*, 1997) and MT_2 knockout mice (Jin *et al.*, 2003) has contributed to reveal these various physiological functions and to attribute them to one or the other receptor type. Recent reviews have focused on the description of these mouse models, the relevance to human disease and physiology, and therapeutic potential (Tosini *et al.*, 2014; Zlotos *et al.*, 2014; Jockers *et al.*, 2016; Liu *et al.*, 2016). In this review, we will focus on novel pharmacological aspects of melatonin receptors, discuss their signalling diversity in the context of system bias and start to unravel the molecular basis for system bias by defining melatonin receptor-associated protein partners. The last section will put the discovery of multiple rare variants of melatonin receptors and associated modification of receptor function in the context of disease risk.

Melatonin receptor pharmacology

State of the art on melatonin receptor ligands

The pharmacological characterization of melatonin receptors started in the late 80s, when melatonin binding sites were detected by using the radiolabelled ligand **2-[^{125}I]-iodomelatonin** (Dubocovich, 1988). The cloning and expression of recombinant melatonin receptors in the 90s allowed further advances in the pharmacological and functional characterization of MT_1 and MT_2 receptors (Reppert *et al.*, 1994, 1995a,b,1996b). In some studies, tritiated melatonin (**[^{3}H]-melatonin**) is also used as a radioligand. However, its low specific activity hampered its broader use despite the fact that it is structurally closer to melatonin than 2-[^{125}I]-iodomelatonin. Of note, a recent study comparing the pharmacology of melatonin receptors in 2-[^{125}I]-iodomelatonin and [^{3}H]-melatonin binding experiments revealed that these radioligands do not behave identically. Whereas [^{3}H]-melatonin detects two melatonin binding sites in both MT_1 and MT_2 receptors, 2-[^{125}I]-iodomelatonin detects only one binding site (Legros *et al.*, 2014). Using G-protein uncoupling agents, the authors concluded that the two binding sites detected by [^{3}H]-melatonin represent two different receptor populations, that is, in their activated and inactivated state, while 2-[^{125}I]-iodomelatonin binds only to activated receptors. Novel iodinated radioligands like **S70254** and **DIV880**, which are the first MT_2-selective radioligands, and **SD6**, an analogue of 2-[^{125}I]-iodomelatonin, were recently added to the tool box (Legros *et al.*, 2013; 2016). Although these are promising ligands, it must be noted that they did not detect melatonin binding sites in some brain areas known to express melatonin receptors, like the SCN, pointing to the need for further improvements of these tools.

Considerable efforts are currently being made to develop fluorescently labelled melatonin receptor ligands. These are promising compounds for investigating the pharmacology and localization of melatonin receptors; they can replace radioligand binding assays and be used to visualize receptors in imaging assays respectively. The ligands described so far include the 7-azamelatonin analogue (Wu *et al.*, 2007), coumarin-based compounds (de la Fuente Revenga *et al.*, 2015) and bodipy-fused analogues (Thireau *et al.*, 2014; Viault *et al.*, 2016; Gbahou *et al.*, 2017). Some of these compounds bind with good affinity (nM range) and non-selectively to MT_1 and MT_2 receptors, but data on their full pharmacological and functional properties are only fragmentary.

Competition binding experiments, mainly with 2-[^{125}I]-iodomelatonin, contributed largely to the identification of melatonin receptor-selective ligands as recently reviewed (Zlotos *et al.*, 2014; Jockers *et al.*, 2016). The competitive non-selective antagonist **luzindole** is still the most used pharmacological tool for characterizing membrane receptor-mediated effects, while the MT_2-selective antagonist 4-phenyl-2-propionamidotetralin (**4P-PDOT**) is the main pharmacological tool used to discriminate between MT_1- and MT_2-mediated effects (IUPHAR database). Reliable MT_1-selective ligands are still unavailable. Recently, pharmacoinformatic approaches have revealed an intriguing class of compounds that are able to bind melatonin receptors with μM affinity: carbamate-derived insecticides (carbaryl and carbofuran) (Popovska-Gorevski *et al.*, 2017). In fact, previous observations indicated that these compounds affect the function of the pineal gland, resulting in altered levels of nocturnal plasma melatonin (Attia *et al.*, 1991).

Table 1

Distribution of MT_1 and MT_2 melatonin receptors expressed in human tissues

		Tissue	Technique	Reference
hMT_1	Brain	Cerebellum	RT-PCR	Mazzucchelli *et al.* (1996)
			In situ hybridization	Al-Ghoul *et al.* (1998)
		Occipital cortex	RT-PCR	Mazzucchelli *et al.*, (1996)
		Parietal cortex	RT-PCR	Mazzucchelli *et al.* (1996)
		Temporal cortex	RT-PCR	Mazzucchelli *et al.* (1996)
		Thalamus	RT-PCR	Mazzucchelli *et al.* (1996)
		Frontal cortex	RT-PCR	Mazzucchelli *et al.* (1996)
		Hippocampus	RT-PCR	Mazzucchelli *et al.* (1996)
			Immunohistochemistry	Savaskan *et al.* (2001)
		SCN	*In situ* hybridization	Weaver and Reppert (1996)
	Peripheral tissues	Retina	Immunocytochemistry	Savaskan *et al.* (2001)
				Savaskan *et al.* (2002)
				Scher *et al.* (2002)
				Scher *et al.* (2003)
		Brown and white adipose tissue	RT-PCR	Brydon *et al.* (2001)
		Fetal kidney	RT-PCR	Drew *et al.* (1998)
		Coronary artery	RT-PCR	Ekmekcioglu *et al.* (2001a,b)
		Granulosa cells	RT-PCR	Soares *et al.* (2003)
				Niles *et al.* (1999)
		Myometrium	RT-PCR, *in-situ* hybridization	Schlabritz-Loutsevitch *et al.* (2003)
		Pancreatic alpha and beta cells	RNA sequencing	Blodgett *et al.* (2015)
		Testis	RT-PCR	Rossi *et al.* (2014)
hMT_2	Brain	Cerebellum	*In situ* hybridization	Al-Ghoul *et al.* (1998)
		Hippocampus	Immunocytochemistry	Savaskan *et al.* (2005)
		SCN	Immunocytochemistry	Wu *et al.* (2013)
	Peripheral tissues	Retina	RT-PCR	Reppert *et al.* (1995a)
			Immunohistochemistry	Savaskan *et al.* (2007)
		Brown and white adipose tissue	RT-PCR	Brydon *et al.* (2001)
		Fetal kidney	RT-PCR	Drew *et al.* (1998)
		Granulosa cells	RT-PCR	Soares *et al.* (2003)
				Niles *et al.* (1999)
		Placental tissues	RT-PCR and Western blot	Lanoix *et al.* (2006)
		Myometrium	*In-situ* hybridization	Schlabritz-Loutsevitch *et al.* (2003)
			RT-PCR	Sharkey *et al.* (2009)
		Pancreatic alpha and beta cells	RNA sequencing	Blodgett *et al.* (2015)
		Testis	RT-PCR	Rossi *et al.* (2014)

Therapeutic applications of melatonin receptor ligands and multi-target-directed ligands

The following melatonin receptor ligands are currently available as marketed drugs for the treatment of conditions linked to circadian dysfunction: **ramelteon** [previously named TAK-375, commercialized as Rozerem® to treat insomnia (Uchikawa *et al.*, 2002; Erman *et al.*, 2006)], **agomelatine** [previously named S20098, commercialized as Valdoxan® to treat depression (de Bodinat *et al.*, 2010; Guardiola-Lemaitre *et al.*, 2014)] and **tasimelteon** (VEC-162) [previously named BMS-214778, commercialized as Hetlioz® to treat sleep and circadian disturbances (Vachharajani *et al.*, 2003; Rajaratnam *et al.*, 2009)]. A recent review summarized the clinical and preclinical effects of these currently marketed drugs targeting melatonin receptors (Liu *et al.*, 2016).

Several recent studies have proposed treatments based on so-called multi-target-directed ligands (Talevi, 2015). Molecules designed to display at least two complementary functions are attractive as (i) they take into account the fact that diseases are often complex multifactorial conditions and, as such, better results are expected; (ii) they abolish

the risk of drug interaction, in which case they can replace the co-administration of two different drugs; and (iii) they facilitate pharmacokinetic aspects, compared with the co-administration condition. Melatonin-derived multi-target-directed ligands were developed mainly for the treatment of Alzheimer's disease (AD) in an attempt to combine the beneficial effects of melatonin as a neuroprotective, antioxidant and anti-amyloidogenic agent with other neuroprotective molecules or with classical anti-cholinesterase inhibitors currently used as the sole treatment for AD. Examples of these hybrid compounds are tacrine–melatonin (Spuch *et al.*, 2010; Zawadzka *et al.*, 2013); melatonin–*N*,*N*-dibenzyl(*N*-methyl)amine hybrid ITH91/IQM157 (Buendia *et al.*, 2015a); (−)-meptazinol–melatonin hybrids (Cheng *et al.*, 2015); curcumin–melatonin (Chojnacki *et al.*, 2014; Gerenu *et al.*, 2015); melatonin–sulforaphane hybrid ITH12674 (Egea *et al.*, 2015) and donepezil–melatonin hybrids (Wang *et al.*, 2016). *In vitro* and *in vivo* characterization studies confirmed that many of these molecules show the expected combined effects, validating the multi-target-directed ligand approach. The melatonin agonist piromelatine (*N*-(2-[5-methoxy-1H-indol-3-yl]ethyl)-4-oxo-4H-pyran-2-carboxamide, or Neu-P11) (Tian *et al.*, 2010), tested in phase I clinical trial to treat insomnia and currently under phase II clinical trial to treat AD (Neurim Pharmaceuticals Ltd, 2011a,b, 2017), can also be classified as multi-target-directed ligand, as it acts also as an agonist at the **5-HT$_{1A}$** and **5-HT$_{1D}$** receptors (He *et al.*, 2013; Liu *et al.*, 2014). Similarly, the anti-depressive melatonin analogue agomelatine (Valdoxan®) is also a multi-target ligand, with agonistic properties at MT_1 and MT_2 receptors and antagonistic properties at the **5-HT$_{2c}$** receptor (Millan *et al.*, 2011).

Biased melatonin receptor ligands

Both melatonin receptors are mainly coupled to the $G\alpha_{i/o}$ proteins and, thus, melatonin classically signals through dampening of the cAMP/PKA pathway. Additional intracellular cascades that are commonly measured include the melatonin-induced activation of MEK/ERK kinases and the recruitment of β-arrestins (Jockers *et al.*, 2016). Studies on biased ligands for melatonin receptors, that is, ligands preferentially modulating one pathway over another as compared with melatonin, are still in their infancy. The first melatonin receptor ligand characterized as a biased ligand was recently described and named ICOA-9 (Gbahou *et al.*, 2017). ICOA-9 shows preferential signalling for the $G_{i/o}$/cAMP pathway over the β-arrestin2 and ERK pathways following human MT_1 receptor activation. Furthermore, the clinically active antidepressant agomelatine shows functional properties on MT_2/5-HT_{2C} heteromers that are biased towards the G_i/cAMP pathway and, thus, distinct from those of melatonin and 5-HT_{2C}-specific antagonists (see next section for more details). Molecular modelling together with molecular docking studies could provide clues about the structural requirements for biased melatonin receptor ligands, similar to the modelling-assisted study that helped to design ligands with MT_1 versus MT_2 receptor selectivity and MT_2-specific antagonistic properties (Pala *et al.*, 2013; Spadoni *et al.*, 2015).

System bias of melatonin receptor pharmacology

The above-mentioned multi-target drugs add a new twist to the system bias of melatonin receptor pharmacology, since it implies a context-dependent efficacy of the ligand depending on the presence or absence of heteromeric receptor complexes. Interestingly, agomelatine not only targets MT_1, MT_2 and 5-HT_{2C} receptors separately but also the heteromeric complex comprising MT_2 and 5-HT_{2C} receptors (Kamal *et al.*, 2015), which introduces a previously underappreciated system bias. In cells expressing only MT_2 receptors, melatonin has no effect on the inositol phosphate (IP) signalling pathway, while it behaves as an agonist at this pathway in the presence of the 5-HT_{2C} receptor. Similarly, agomelatine has no effect on this pathway in cells expressing only MT_2 receptors, but it antagonizes melatonin-induced IP production in cells co-expressing MT_2 and 5-HT_{2C} receptors (Kamal *et al.*, 2015). In addition, whereas melatonin shows agonistic properties on the cAMP and IP pathway, agomelatine is an agonist of the cAMP pathway but a neutral antagonist of the IP pathway compared with melatonin in cells expressing the MT_2/5-HT_{2C} heteromer. Conversely, luzindole and 4P-PDOT, which are competitive antagonists of MT_1 and MT_2 receptors in the absence of the 5-HT_{2C} receptor, behave as agonists of the cAMP pathway and full or partial agonists, respectively, of the IP pathway in cells expressing the MT_2/5-HT_{2C} heteromer.

Evidence for the existence of system bias in melatonin receptor pharmacology also comes from studies showing that ligand efficacy depends on the cell context, on the receptor expression levels in a given tissue and on their active or inactive state (Legros *et al.*, 2014). For example, luzindole and 4P-PDOT (at high concentrations) are usually competitive melatonin receptor antagonists, but behave as inverse agonists at MT_1 receptors in the presence of constitutively activated receptors, as shown in rat artery cells expressing endogenous MT_1 receptors (Ersahin *et al.*, 2002). Constitutive activation was also observed for MT_1 and MT_2 receptors in transfected CHO and Neuro2A cell lines, and MT_2-specific inverse agonists (UCM 549 and UCM 724) effectively decreased the constitutive activity of MT_2 receptors (Devavry *et al.*, 2012). The pharmacological properties of the competitive melatonin receptor antagonist 4P-PDOT seem to be even more complex, as not only antagonistic and inverse agonistic effects have been described, as mentioned before, but also partial agonistic activity is also reported for native and recombinant MT_2 receptors (Nonno *et al.*, 1999; Ayoub *et al.*, 2002; Dubocovich *et al.*, 2003). The impact of the cell context on the pharmacological properties of melatonin receptors is further confirmed by the study of Logez *et al.* (2014). They observed a marked decrease in ligand binding affinities of recombinant human MT_1 receptors when expressed in the eukaryotic microorganism *Pichia pastoris* compared with MT_1 receptors expressed in CHO cells. Intriguingly, after purifying MT_1 receptors from *P. pastoris*, the pharmacological profile of the receptor resembled that observed in CHO membranes (Logez *et al.*, 2014). The authors suspect that differences in the membrane lipid composition, most likely the cholesterol content, between these two cell types are at the origin of the differences observed in the pharmacological profiles.

Collectively, cell context-dependent receptor expression, receptor heterodimerization and context- and ligand-

dependent bias are among the main factors underlying system bias and are proven to be relevant to the elucidation of the pharmacology of melatonin receptors.

Melatonin receptor signalling

Common aspects of melatonin receptor signalling

As yet, the intrinsic affinity of melatonin receptors for different G proteins has not been systematically determined. The G-protein coupling profile also depends on the relative expression levels of the different G proteins in a given cellular context, which accounts for the system bias of melatonin receptor pharmacology. In most experimental systems, both MT_1 and MT_2 receptors are mainly coupled to G_i proteins, thus leading to inhibition of AC activity (Figure 1). A more detailed analysis in HEK293 cells shows that MT_1 receptors co-immunoprecipitate preferentially with $G\alpha_{i2}$ and $G\alpha_{i3}$ proteins and to a lesser extent to $G_{q/11}$, while no coupling to $G\alpha_{i1}$, $G\alpha_z$, $G\alpha_o$, $G\alpha_{12}$ or $G\alpha_s$ was detected (Brydon *et al.*, 1999b). An illustrative example of system bias is the $G\alpha_{16}$ protein, which is exclusively expressed in haematopoietic cells (Amatruda *et al.*, 1991). Co-transfection of MT_1 or MT_2 receptors together with $G\alpha_{16}$ in COS-7 cells leads to the potentiation of melatonin signalling through the JNK pathway, indicating coupling of both melatonin receptors to $G\alpha_{16}$ (Chan *et al.*, 2002). In addition to the frequently observed modulation of cAMP levels by melatonin receptors, modulation of diacylglycerol, inositol trisphosphate and Ca^{2+} levels have been observed in a cell context-dependent manner (Brydon *et al.*, 1999a,b). Tissues and cells in which $G_{q/11}$ coupling to endogenously expressed melatonin receptors have been observed include the myometrium (Steffens *et al.*, 2003), prostate epithelial cells (Shiu *et al.*, 2010), pancreatic cells (Bähr *et al.*, 2012) and human mesenchymal stem cells (Lee *et al.*, 2014), in addition to non-mammalian cells (Hotta *et al.*, 2000) and cells expressing recombinant receptors (MacKenzie *et al.*, 2002).

Melatonin can also regulate ion channels, and multiple pathways seem to be involved. Melatonin modulates muscle contractile responses in arteries (Geary *et al.*, 1998; Masana *et al.*, 2002) and in the myometrium (Steffens *et al.*, 2003) by regulating the activity of large-conductance Ca^{2+}-activated K^+ (**BK_{Ca}**, also known as $K_{Ca}1.1$) channels. In the myometrium, the modulation of BK_{Ca} channels by melatonin was shown to be dependent on the activation of both G_q/PLC/Ca^{2+} and G_i/cAMP/PKA pathways. Interestingly, the final outcome of the effect of melatonin on BK_{Ca} activity depends on the physiological context, as opposite effects are observed in myocytes obtained from pregnant and non-pregnant rats (Steffens *et al.*, 2003). At the transcriptional level, melatonin signalling typically inhibits the transcriptional factor cAMP-responsive element binding (CREB) and activates the transcription of genes under the control of the ERK pathway. Up to now, the major difference between MT_1 and MT_2 receptors at the signalling level concerns their ability to inhibit cGMP production, which is only observed in MT_2-transfected cells (Petit *et al.*, 1999). This effect was confirmed in human non-pigmented ciliary epithelial cells expressing endogenous MT_2 receptors (Dortch-Carnes and Tosini, 2013). The general signalling pathways triggered by melatonin receptors are depicted in Figure 1, but the impact of system bias should be kept in mind. We next present further melatonin functions for which at least some of the signalling intermediates have been described, thus expanding the molecular components of melatonin receptor signalling.

Diversity of melatonin's effects and signalling cascades – further evidence of system bias

The regulation of circadian rhythms by melatonin has been extensively studied (Dubocovich *et al.*, 2010). Melatonin has been shown to affect the firing rate of hypothalamic suprachiasmatic nucleus (SCN) neurons, which constitutes the master clock in mammals. This effect is mediated by both receptors in a G_i-dependent, but cAMP-independent manner. For MT_1 receptors it involves the activation of **G protein-coupled inwardly rectifying potassium channels**, like $K_{ir}3$ (Nelson *et al.*, 1996; van den Top *et al.*, 2001; Hablitz *et al.*, 2015), while the MT_2-induced phase advance in neuronal activity involves the PKC signalling pathway (McArthur *et al.*, 1997; Hunt *et al.*, 2001). In the presence of pituitary AC activating peptide (PACAP)-induced cAMP production, both receptors can modulate neuronal activity by the G_i/cAMP pathway (Jin *et al.*, 2003). Melatonin regulation of clock gene expression has been reported in the SCN and both MT_1 and MT_2 receptors are involved in this effect (Pfeffer *et al.*, 2012; Nagy *et al.*, 2015; Kandalepas *et al.*, 2016). In the striatum, melatonin has been reported to modulate clock gene expression through MT_1 receptors in a G_i-dependent manner (Imbesi *et al.*, 2009). In cerebellar Purkinje cells, the neuronal firing rate is modulated by MT_1 receptors through inhibition of **P-type Ca^{2+} channels** ($Ca_v2.1$) in a G_i/$G\beta\gamma$/PI3K/PKCδ signalling-dependent manner (Zhang *et al.*, 2015). Together with the SCN, the hypophyseal *pars tuberalis* is the main target of melatonin involved in its synchronizing effects. Melatonin transduces photic information through MT_1 receptors by regulating the expression of *mPer1*, *mCry1*, *Clock* and *Bmal1* genes through a heterologous repressive/sensitization of the cAMP pathway that requires not only MT_1 receptors but also the adenosine A_{2B} receptor (von Ball *et al.*, 2002; Dardente *et al.*, 2003; von Gall *et al.*, 2005; Wood and Loudon, 2014). The regulator of G-protein signalling (RGS)4 (Dupre *et al.*, 2011) and the basic helix-loop-helix Per-Arnt-Sim domain transcription factor NPAS4 are also suggested to participate in this signalling cascade (West *et al.*, 2013). Although the circadian machinery is present in every cell, the effect of melatonin on rhythmic clock gene expression in other tissues is not clear and seems to be cell type-dependent (Muhlbauer *et al.*, 2009; Owino *et al.*, 2016). The precise mechanism underlying the effect of melatonin on clock genes is not well defined and might involve transcriptional and post-translational modulation of clock proteins (reviewed by Vriend and Reiter, 2015).

Many studies have demonstrated that melatonin plays an important role in regulating different aspects of retinal physiology. The clock machinery of the retina is responsive to melatonin, and both receptors are involved, but the precise signalling pathway has not, as yet, been elucidated. In MT_1 receptor knockout mice, the rhythmic expression of *Per1* was not abolished, but the phase was significantly affected

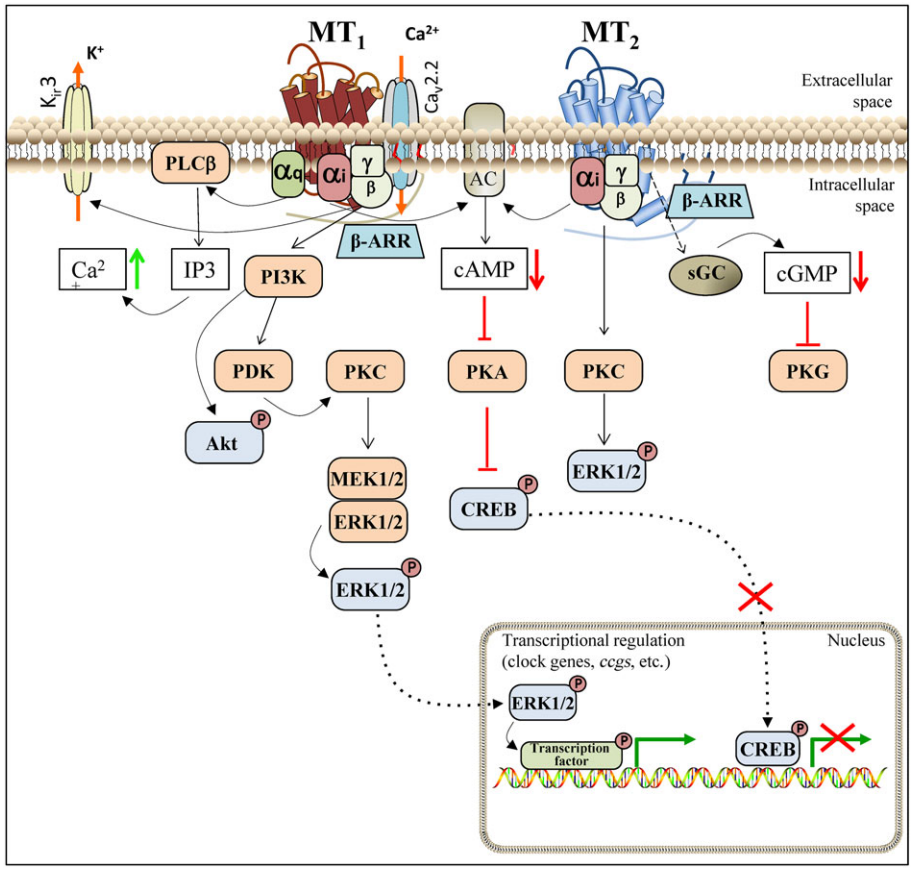

Figure 1

Melatonin receptor signalling pathways. Melatonin activation of MT_1 receptors triggers $G\alpha_i$ activation, decreasing the levels of the secondary messanger cAMP, and $G\beta\gamma$-dependent activation of PI3K/Akt, PKC and ERK pathways. MT_1 coupling to Gq leads to PLC activation and increase in intracellular Ca^{2+}. Melatonin-induced modulation of neuronal action potential is mediated by MT_1-dependent activation of the potassium and calcium ion channels ($K_{ir}3$ and $Ca_v2.2$). The physical interaction of MT_1 receptors with $Ca_v2.2$ channels tonically inhibits $Ca_v2.2$-mediated calcium entry through $G\beta\gamma$ subunits. Melatonin activation of MT_2 receptors triggers $G\alpha_i$-dependent cAMP and ERK signalling pathways and inhibits cGMP levels. Melatonin induced β-arrestin recruitment to both MT_1 and MT_2 receptors, but β-arrestin-dependent down-streaming signalling is not yet reported. See text for details. β-ARR, β-arrestin; $Ca_v2.2$, voltage-gated calcium channel; *ccgs*, clock-controlled genes; CREB, cAMP-responsive element binding; $K_{ir}3$, G protein-coupled inwardly rectifying potassium channel; sGC, soluble GC.

(Dinet *et al.*, 2007). Significant changes in the pattern of expression of other clock genes, as well as clock-controlled genes (ccgs, like *cfos*) were also observed in melatonin receptor knockout mice (Hiragaki *et al.*, 2014; Kunst *et al.*, 2015). Melatonin controls the retinal light sensitivity at night (Baba *et al.*, 2013), an effect that was shown to depend on MT_1/MT_2 heteromers (further discussed in the Melatonin receptor oligomers section), which preferentially activate the $G_q/PLC/Ca^{2+}$ pathway (Baba *et al.*, 2013), while regulation of photoreceptor viability is believed to depend on the survival-related Akt/FOXO1 signalling pathway (Gianesini *et al.*, 2016).

In addition to retinal cells, melatonin also modulates the viability of neurons under physiological and pathological conditions. The neuroprotective and anti-apoptotic properties of endogenous and exogenous melatonin have been extensively investigated, and different signalling pathways underlie these effects. In neural stem cells, the effect of melatonin on cell survival, maturation and differentiation is melatonin receptor-dependent, as it is prevented by the competitive melatonin receptor antagonist luzindole (Ramirez-Rodriguez *et al.*, 2009; Tocharus *et al.*, 2014; Chu *et al.*, 2016; Ortiz-Lopez *et al.*, 2016). In pluripotent stem cells melatonin-induced neural differentiation involves the PI3K/Akt pathway and is also blocked by luzindole (Shu *et al.*, 2016). However, prolonged exposure of embryonic stem cells to melatonin favours the pluripotency state of the cells in a MT_1-dependent manner that involves a synergism between the effect of the PI3K/Akt and ERK pathways that results in the up-regulation of the glucose transporter GLUT1 (Wu *et al.*, 2017). In an *in vivo* rat model of neuro-inflammation, endogenous melatonin protects cerebellar neurons from LPS toxicity, while neuronal death is observed in the presence of the competitive melatonin receptor antagonist luzindole (Pinato *et al.*, 2015). Similarly, Wang *et al.* (2011) observed that neurons were more vulnerable to cell death in the presence of luzindole and in MT_1-silenced cells. In ischaemia/reperfusion models, the protective effect of melatonin relies on its anti-apoptotic and antioxidant effects, as it is known to up-regulate several antioxidant enzymes,

including SOD1 and glutathione peroxidase (Parada *et al.*, 2014; O'Neal-Moffitt *et al.*, 2015; Ramos *et al.*, 2017). In cerebral ischaemia, the protective effect of melatonin and agomelatine was linked to activation of the nuclear factor erythroid-related factor 2 (Nrf2), which regulates the expression of antioxidant enzymes (Ding *et al.*, 2014; Chumboatong *et al.*, 2017). In *in vitro* and *in vivo* ischaemic models, the multi-target-directed 5-HT and melatonin receptor Neu-P11 ligand promoted neuronal survival through the activation of PI3K/Akt, ERK and JAK2 pathways (Buendia *et al.*, 2015b) (Figure 2).

A number of observations suggest that the antioxidant and anti-apoptotic effects of melatonin depend largely on mitochondrial function and dynamics (Tan *et al.*, 2016). Melatonin has been reported to modulate the expression levels and localization of Bax and Bcl-2 proteins and to inhibit the release of cytochrome c and the activation of caspase-3 (Radogna *et al.*, 2008; Wang *et al.*, 2009; Luchetti *et al.*, 2010). The JAK2/STAT3 pathway is suspected to mediate melatonin-induced Bax/Bcl-2 translocation in cardiomyocytes (Yang *et al.*, 2013), while ERK activation and p38 MAPK inhibition were proposed to mediate the anti-apoptotic effect of melatonin in monocytes (Luchetti *et al.*, 2009). Additional mitochondria-associated signalling cascades activated by melatonin include activation of **sirtuin histone deacetylases** (SIRTs) (reviewed by Mayo *et al.*, 2017) through AMPK/SIRT3/SOD2 and SIRT1/PPAR-γ coactivator (PGC-1α), as shown in hepatocytes (Guo *et al.*, 2014; Chen *et al.*, 2015; Pi *et al.*, 2015). Of note, the transcription factor PGC-1α is also regulated by MT_1 receptors in the retina (Kunst *et al.*, 2015). Melatonin signalling through SIRT might also underlie the well-known anti-inflammatory action of melatonin through inhibition of the inflammatory transcription factor NF-κB (Tajes *et al.*, 2009; Zhao *et al.*, 2017). In mouse models of neurodegenerative diseases, such as AD, amyotrophic lateral sclerosis and Huntington's disease, the neuroprotective effect of melatonin was linked to MT_1-dependent modulation of mitochondrial function (Dragicevic *et al.*, 2011; Wang *et al.*, 2011; Zhang *et al.*, 2013). Interestingly, the inhibitory effect of melatonin on cytochrome c release could be reproduced in purified brain mitochondria, presumably through mitochondrial MT_1 receptors, as suggested by the authors (Wang *et al.*, 2011). Recently, further evidence for the intriguing mitochondrial localization of MT_1 receptors was obtained by using the first cell-impermeable melatonin receptor agonist, which allowed us to discriminate between MT_1-triggered G_i/cAMP signalling at the cell surface and in mitochondria (Gbahou *et al.*, 2017). Whether the neuroprotective effect of melatonin linked to mitochondrial function is due to mitochondrial MT_1 signalling is currently under investigation.

In addition to the above-mentioned examples, the impact of system bias on melatonin receptor signalling can also be

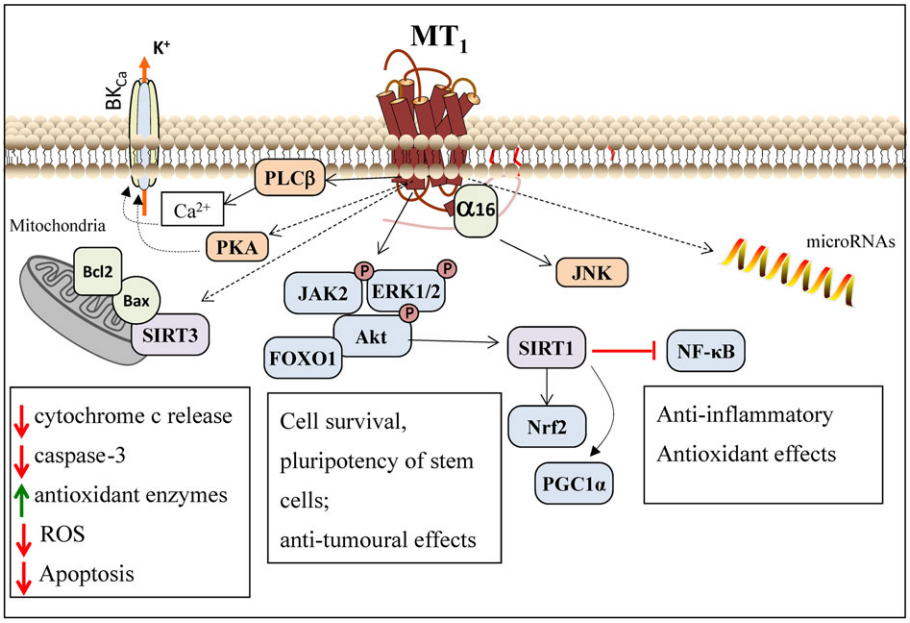

Figure 2

Extended, context-specific melatonin receptor signalling pathways. Depending on the cell type or the presence of cell stressors, melatonin can activate additional melatonin receptor-dependent signalling cascades. These pathways have been reported mainly for MT_1 receptors, but the participation of MT_2 receptors cannot be excluded. Melatonin modulation of mitochondrial function is reported under oxidative stress condition and in neurodegenerative diseases. Proposed signalling pathways involve the regulation of the activity and/or translocation of Bcl2/Bax and SIRT proteins. Activation of JAK2, ERK and the Akt/FOXO1 complex are suggested to mediate melatonin-induced cell survival and to modulate pluripotency/differentiation of stem cells, while melatonin-induced inhibition of these pathways is reported in cancer cells. MT_1-dependent activation of SIRT1 might underlie melatonin anti-inflammatory and anti-oxidative effects through regulation of transcription factors like Nrf2, PGC1α and NF-κB. MT_1-coupling to G_{16} protein occurs in haematopoietic cells and triggers the JNK pathway. In several cell types, including cancer cells, melatonin is reported to modulate the expression of different miRNAs. See text for details. SIRT, sirtuin.

nicely exemplified in the cancer field, where the repertoire of signalling pathways modulated by melatonin is highly context-specific. In general, melatonin is reported to display anti-tumoural properties, inhibiting proliferation and inducing apoptosis. In breast cancer models, the anti-tumoural effect of melatonin appears to involve mainly MT_1 receptors through inhibition of the phosphorylation of signalling molecules such as AKT, ERK and PKC (Hill *et al.*, 2011; 2015). In these cells, melatonin has also been reported to activate the p53 DNA-protective pathway in a receptor-dependent manner (Santoro *et al.*, 2013), while in ovarian cancer, melatonin inhibits Akt, p38 MAPK and mTOR signalling (Ferreira *et al.*, 2014).

New players contributing to system bias of melatonin receptor function

MicroRNAs

Additional complexity to the study of melatonin receptors signalling emerges when considering the growing number of reports pointing to the role of microRNAs (miRNA) in mediating the effects of melatonin. In prostate cancer cells, the anti-angiogenic effect of melatonin was linked to an up-regulation of miRNA3195 and miRNA374b (Sohn *et al.*, 2015). MiR-24 is a miRNA often up-regulated in several types of cancer, and melatonin was able to down-regulate it in a luzindole-sensitive manner (Mori *et al.*, 2016). Regulation of miRNAs by melatonin has been also demonstrated in hepatocytes (Kim *et al.*, 2015; 2017) and neurons (Carloni *et al.*, 2016). Interestingly, miRNAs can also regulate the expression of MT_1 receptors (Zhu *et al.*, 2014). It has recently been reported that the expression of miRNAs can vary in a daily pattern (Marcola *et al.*, 2016), which introduces an additional bias regarding the time when data are collected. Because miRNAs are highly context-dependent, it is likely that they greatly contribute to the system bias on melatonin receptors signalling. Altogether, an extended melatonin receptor signalling network is emerging from these studies in the light of the system bias concept (Figure 2).

Melatonin receptor oligomers

The work from Ayoub *et al.* (2002) was the first to propose that, similar to other GPCRs, melatonin receptors could exist as homomers and/or heteromers. By BRET experiments using transfected HEK293 cells, we demonstrated that melatonin receptors form homomers and heteromers in living cells, with prevalence for the formation of the heteromers (Ayoub *et al.*, 2002; 2004). Melatonin activation did not have any apparent effect on the oligomerization state of the receptors. Importantly, the heteromer showed a distinct pharmacological profile in BRET experiments compared with MT_2 homomers, with the competitive antagonist luzindole being 100 times more potent on heteromers (Ayoub *et al.*, 2004). Both binding sites seem to be preserved in the heteromer when inspected individually and are not subject to negative cooperativity (Ayoub *et al.*, 2004). Although many GPCRs have a natural tendency to oligomerize (Ferre *et al.*, 2014), such oligomers appear to be less abundant in tissues at natural expression levels, and their physiological relevance has been proven only in some cases. MT_1/MT_2 heteromers were detected in retinal photoreceptor cells and were shown to be key players in improving retinal light sensitivity at night (Baba *et al.*, 2013). The *in vitro* characterization of the heteromer revealed that the PKC signalling pathway is potentiated by melatonin under this condition and, indeed, the *in vivo* effect is also PKC-dependent (Baba *et al.*, 2013). Inasmuch as many tissues express both melatonin receptors, including the SCN, the involvement of MT_1/MT_2 heteromers in mediating melatonin's effects deserves further investigation. Interestingly, different melatonin signalling patterns are detected in cerebellar granular cells expressing endogenous MT_1 and MT_2 receptors. In this cellular context, melatonin at a low nM concentration inhibits rather than stimulates ERK and Akt pathways, while a stimulatory response is detected if either receptor is silenced. Conversely, melatonin decreases forskolin-simulated cAMP production only in cells expressing both MT_1 and MT_2 receptors, while no effect is detected in cerebellar granular cells expressing only one type of melatonin receptor (Imbesi *et al.*, 2008). It is likely that the responsiveness of these cells to melatonin relies on the MT_1/MT_2 heteromer.

The orphan receptor of the melatonin receptor family, GPR50, has also been demonstrated to engage in oligomeric complexes with MT_1 and MT_2 receptors (Levoye *et al.*, 2006). Contrary to what was observed for MT_1/MT_2 heteromers, in this case, the dimer formation markedly altered ligand binding and signalling properties of MT_1, but not of MT_2 receptors, as melatonin binding and G_i protein and ß-arrestin coupling of MT_1 receptors are lost in the MT_1/GPR50 heterodimer. The negative modulation of MT_1 receptors by GPR50 was confirmed in hCMEC/D3 cells expressing both receptors endogenously, as melatonin signalling was observed only after GPR50 silencing (Levoye *et al.*, 2006). The physiological relevance of the MT_1/GPR50 heterodimer, as well as the regulatory factors of their association and dissociation *in vivo*, remain to be elucidated. Nevertheless, the fact that GPR50 is expressed in the pituitary gland, in several hypothalamic nuclei and in the median eminence, which are main loci of melatonin's action, as well as in other central areas (Batailler *et al.*, 2012), implies that a GPR50-dependent regulation of melatonin receptors signalling might be physiologically relevant.

As mentioned before, the pharmacological properties of agomelatine lead to the investigation of the existence of heteromers comprising melatonin and 5-HT receptors. In transfected HEK293 cells, it was observed that both MT_1 and MT_2 receptors are able to associate with the 5-HT_{2c} receptor in a heteromeric complex (Kamal *et al.*, 2015). The pharmacology of MT_2 receptors seems to be altered in the heteromer, as melatonin activates not only G_i-dependent signalling but also G_q/PLC signalling, which is not observed in cells expressing the MT_2 receptor alone (Kamal *et al.*, 2015). We proposed a transactivation model in which the melatonin-activated MT_2 receptor is able to allosterically transactivate 5-HT_{2c}-dependent G_q signalling. These heteromers were also targeted by agomelatine suggesting that MT_2/5-HT_{2c} heteromers might participate in the antidepressant effect of this drug. The biased pharmacology of melatonin receptor dimers is shown in Figure 3.

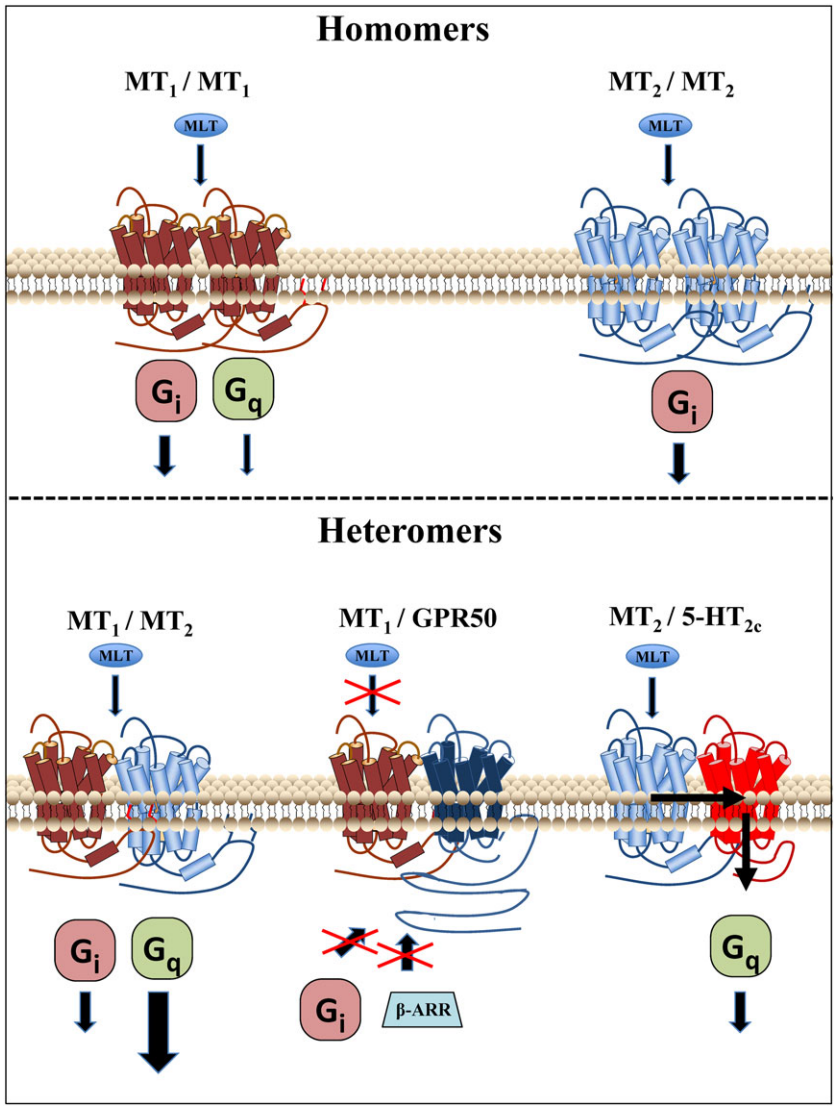

Figure 3

Signalling of melatonin receptor homomers and heteromers. Melatonin activation of MT_1/MT_1 homomers triggers intracellular signalling predominantly through the G_i pathway over the G_q pathway, while MT_2/MT_2 homomers signal exclusively through G_i proteins. In the MT_1/MT_2 heteromer, melatonin signalling is biased towards G_q activation over G_i. When dimerizing with GPR50, the MT_1 receptor loses its ability to bind melatonin, to trigger G_i signalling and to recruit β-arrestin (β-ARR). Melatonin activation of the $MT_2/5\text{-}HT_{2c}$ heteromer triggers $5\text{-}HT_{2C}$ receptor dependent G_q signalling through a MT_2 receptor transactivation mechanism. See text for details. MLT, melatonin.

Melatonin receptor-associated protein complexes

Formation of receptor heteromers is only one of the possible ways that GPCRs have to shape their cellular microenvironment to ultimately determine the signalling outcome. Other proteins that might be constitutive or agonist-induced components of these receptor-associated complexes have also been identified for melatonin receptors. By combining different proteomic and genomic approaches and different biological resources expressing endogenous melatonin receptors, an interactome of MT_1 and MT_2 receptors composed of 366 individual proteins was built (Figure 4) (Daulat *et al.*, 2007; Benleulmi-Chaachoua *et al.*, 2016). This represents one of the most complete and diverse GPCR interactomes currently available. Out of the 366 interactors, only 52 were identical between the two receptors. Many of these common interactors belong to the family of small G proteins (Rab and Rho GTPases), heterotrimeric G proteins, molecular chaperones (calnexin and calreticulin), cytosqueleton components (filamin, myosin, etc.) and ubiquitin ligases. This suggests common functions and highlights the previously underappreciated link between melatonin receptors and small G proteins and cytosqueleton organization that warrants further attention.

MT_1 and MT_2 receptors are known to undergo agonist-dependent and -independent internalization (Gerdin *et al.*, 2003; Guillaume *et al.*, 2008). The melatonin receptor interactome contains trafficking proteins such as caveolin,

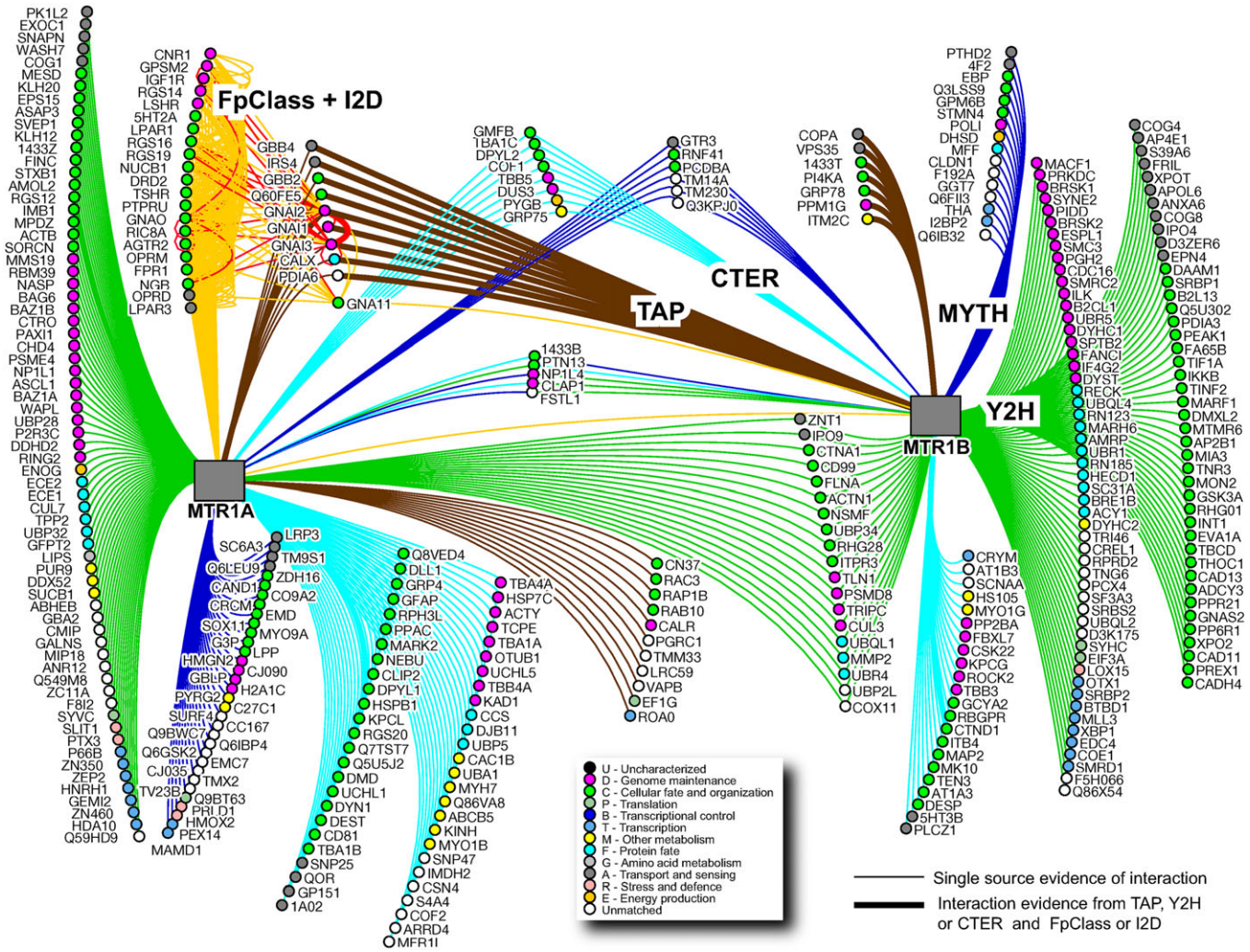

Figure 4

Melatonin receptors interactome. MTR1A (MT_1)- and MTR1B (MT_2)-interacting proteins were identified in 20 different screens and are clustered based on the different identification methods: dark blue for the MYTH, blue for Cter peptide purification, green for the Y2H and brown for the TAP methods. Thick lines correspond to confirmed protein–protein interactions and node colours refer to predicted gene ontology biological function. See text for details. CTER, carboxyl terminus peptide affinity chromatography; MYTH, membrane yeast two-hybrid; TAP, tandem affinity purification; Y2H, yeast two-hybrid. Modified from Benleulmi-Chaachoua *et al.* (2016), with permission.

dynamin 1, AP2 and AP4 adaptor proteins that act in concert with GPCRs kinases and ß-arrestins to promote receptor internalization (Levoye *et al.*, 2006; Bondi *et al.*, 2008; Maurice *et al.*, 2008).

The interactome also revealed that most of the interactions are unique for each receptor (168 and 143 for MT_1 and MT_2 respectively). This result was unexpected given the fact that previous studies revealed only few functional differences between MT_1 and MT_2 receptors. These novel results will be a rich source of investigation for the next few years to unravel and understand the functional differences between both receptors. Among the possible new functions are links with several ion transporters/channels like the electroneutral potassium-chloride co-transporter 1, the zinc transporters and the electroneutral Na/HCO_3 co-transporter in the MT_2 interactome. Another remarkable finding was the exclusive presence of several synaptic proteins in the MT_1 interactome such as synapsin, SNAP25 and 47, the voltage-gated calcium channel $Ca_v2.2$, Munc-18, rabphilin and snapin. The absence of these proteins from the MT_2 interactome suggested differences in the subcellular localization of MT_1 and MT_2 receptors in neurons, a notion that was confirmed in primary hippocampal neurons. The different localization of melatonin receptors in neurons is likely to increase our understanding of the different roles of both receptors in the brain and, at the same, adds a not yet appreciated spatial bias to melatonin receptor function. The interactome also provides some clues as to how the different localization of both receptors is achieved as the MT_1, but not the MT_2 receptor, interacts with kinesin-1, which has been shown to transport the Na^+ channel to axons (Barry *et al.*, 2014). The interaction of MT_1 receptors with the $Ca_v2.2$ channel

was shown to be of functional importance as *in vitro* experiments in CHO cells showed that the presence of MT_1 receptors tonically inhibits the $Ca_v2.2$-promoted calcium current through Gβγ subunits (Benleulmi-Chaachoua *et al.*, 2016). Indeed, voltage-dependent inhibition involving direct binding of Gβγ subunit to the channel (Zamponi and Currie, 2013) is the most widespread mechanism by which GPCRs regulate voltage-gated calcium channels. Localization of MT_1 receptors in the presynaptic membrane suggests its involvement in synaptic functions such as neurotransmission. This conclusion corroborates earlier reports suggesting a possible influence of melatonin on neurotransmitter release and uptake in some brain regions, like the ventral hippocampus, medulla pons, preoptic area and median and posterior hypothalamus but not in others (Cardinali *et al.*, 1975; Zisapel and Laudon, 1982). This new input from the interactome analysis will renew the interest in the possible role of MT_1 receptors in neurotransmitter regulation.

Among the best-characterized interactors of the MT_1 receptor is RGS20 (Maurice *et al.*, 2010) and the multi-PDZ domain protein, MUPP1 (Guillaume *et al.*, 2008). Whereas RGS20 regulates the speed of G_i-protein signalling of MT_1 receptors by accelerating the activation kinetics of $K_{ir}3$ channels, the expression of MUPP1 is obligatory for MT_1 receptors to inhibit cAMP production by a yet poorly defined mechanism. Altogether, these data demonstrate the tremendous influence of melatonin receptor-associated proteins on their function and underline the necessity to define the interactome of melatonin receptors in a given cell type to fully understand the functional outcome of melatonin stimulation in a given tissue.

Melatonin receptor variants

Receptor variants introduce a bias at the level of the receptor that contributes to inter-individual differences in receptor function, which are suspected to contribute to the risk of common diseases. Genetic variation may also have important consequences on drug action. This variation may occur at the level of different ethnic groups (typically for frequent variants) or at the level of individuals (typically for rare or very rare variants). Consequently, information of the existence of receptor variants within cohorts for clinical trials helps to stratify and homogenize the cohorts to decrease their genetic variability and to help improve the outcome for clinical trials.

Recent large-scale sequencing studies revealed considerable inter-individual genetic variability in GPCR coding genes. In humans, an average of 32 non-synonymous variants has been estimated to exist per GPCR in a sample of 10 000 individuals (Nelson *et al.*, 2012; Karamitri and Jockers, 2014). In the case of melatonin receptors, 8 and 42 non-synonymous variants have been demonstrated to occur in the *MTNR1A* and *MTNR1B* genes coding for the MT_1 and MT_2 receptor respectively (Jockers *et al.*, 2016). These numbers are likely to increase with the increased number of sequenced human genomes and targeted exon sequencing. Based on reports indicating that an alteration in melatonin synthesis is associated with autism spectrum disorders (ASDs), non-synonymous variants have been identified in the *MTNR1A* and *MTNR1B* genes in 300 ASD patients and a matched control population. No significant difference in the prevalence of these variants was found indicating that they do not contribute to ASD risk (Chaste *et al.*, 2010). A similar conclusion was reached after sequencing of *MTNR1A* and *MTNR1B* in individuals with attention deficit hyperactivity disorder (ADHD; Chaste *et al.*, 2011). From a pharmacological point of view, it is important to note that the majority of the 16 non-synonymous variants identified in these studies showed altered receptor function with some of them showing a biased signalling profile compared with the wild-type receptor (Chaste *et al.*, 2010). This was the first report to show that variability of melatonin action does exist at the receptor level.

Inspired by genome-wide association studies revealing a robust association of the minor allele of the common rs10830963 variant located in the intron of *MTNR1B* with increased type 2 diabetes (T2D) risk (Bouatia-Naji *et al.*, 2009; Lyssenko *et al.*, 2009), the two exons of the *MTNR1B* gene were sequenced in 7632 individuals including 2186 individuals with T2D (Bonnefond *et al.*, 2012). Forty non-synonymous MT_2 variants were identified including 38 rare and the two common variants. Those variants with a loss-of-function phenotype, but not the functionally neutral ones, were associated with increased T2D risk, establishing for the first time a link between melatonin receptor function and the risk for a common disease such as T2D. Subsequent studies that attempted to understand the functional basis of the association of the common rs10830963 variant with T2D risk indicated that risk allele carriers express two to four times more *MTNR1B* mRNA in their human pancreatic islets (van de Bunt *et al.*, 2015). Taken together with other experimental findings, a model was proposed based on the assumption that MT_2 protein levels are increased, exaggerating the putative inhibitory effect of melatonin on pancreatic insulin production in risk allele carriers (Tuomi *et al.*, 2016). Obviously, this hypothesis contrasts with the conclusion drawn from rare loss-of-function variants, namely, that defective melatonin receptor function is associated with T2D risk. This apparent controversy is discussed in several recent commentaries (Bonnefond *et al.*, 2016; Mulder, 2017). Drawing the right conclusions will be of relevance for human health, as it will be important to know whether melatonin supplementation (as practised by millions of people in the world) is beneficial or detrimental in terms of glucose homeostasis and T2D risk. From a pharmacological point of view, functional evidence for the expression of MT_2 receptors in human pancreatic beta cells is weak. The MT_2 receptor has not been detected at the protein level, and mRNA expression is only detectable in less than 5% of the cells at very low (close to background) levels (Segerstolpe *et al.*, 2016; Thomsen *et al.*, 2016). Evidence for the inhibitory effect of melatonin on cAMP levels and insulin production is substantial in rodents at the cellular and animal level (Peschke *et al.*, 2007); however, conflicting results are reported in human cells with some studies even showing a stimulation/potentiation of insulin production by melatonin (Ramracheya *et al.*, 2008; Costes *et al.*, 2015). It is important to note that the physiological effect of melatonin on metabolism is expected to be fundamentally different as diurnal humans are day active, whereas nocturnal

rodents are night active, even though melatonin is always secreted during the night. In the light of this inconclusive evidence, further hypotheses have to be explored to understand the effect of melatonin on glucose homeostasis in humans. This includes the search for further functions of melatonin in pancreatic beta cells, such as the recently reported stimulatory role of melatonin on human beta-cell survival (Costes *et al.*, 2015). Whether these effects are indeed mediated through melatonin receptors or through novel pharmacological units, such as heteromeric complexes, has to be investigated. Melatonin target tissues, other than pancreatic beta cells, like the brain or the liver and adipose tissues will have to be considered. Furthermore, focusing on the inhibitory effect of melatonin receptors on the cAMP pathway might also be too restrictive and other G protein- and β-arrestin-dependent signalling events might be more relevant.

In conclusion, the currently available data show that melatonin receptor variants exist in the human population and that they are of relevance for major common diseases. However, we are only at the beginning of our understanding of the full impact of such variants on human health. An expansion of future studies towards the *MTNR1A* gene and other diseases like sleep and circadian rhythm disorders represents an interesting field of future research.

Conclusion and perspectives

Multiple functions have been attributed to melatonin receptors that are transmitted by the activation of a large diversity of signalling pathways. Current knowledge clearly indicates that the signalling profile of melatonin receptors is highly cell- and tissue-dependent, arguing for the existence of system bias as an important determinant of the functional outcome of melatonin receptor signalling. This highly complex arrangement makes it difficult to transpose functional properties described in one cell context into another. It also implies that the exogenous expression of recombinant receptors might be only of limited predictive value for the signalling properties of endogenous melatonin receptors in a given tissue. Interesting areas of future research are the detailed investigation of the intriguing localization of melatonin receptors in intracellular compartments such as mitochondria, the widespread formation of melatonin receptor heteromers and the development of novel generations of multi-target-directed ligands. New radioactive and fluorescently labelled tracer molecules are likely to detect further activation states of melatonin receptors that will be highly informative in defining new melatonin receptor complexes. Finally, the generation of biased ligands for melatonin receptors is still in its infancy but warrants further attention given the huge expectation of these compounds for therapeutic application in terms of signalling specificity and reduced side effects.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b,c).

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Conflict of interest

The authors declare no conflicts of interest.

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