MELATONIN IN HEALTH AND DISEASE

EDITED BY: Ralf Jockers and James M. Olcese
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frontiers Research Topics

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MELATONIN IN HEALTH AND DISEASE

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EDITORIAL
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Editorial: Melatonin in Health and Disease

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Editorial on the Research Topic

Melatonin in Health and Disease

Melatonin was discovered more than 60 years ago as N-acetyl-5-methoxytryptamine, a derivative of tryptophan that is structurally related to serotonin. Since then, an intense research activity developed around this small molecule to which the authors of this Research Topic made seminal contributions.

The first key question in the field was: "Where does melatonin come from or how is it synthesized?". Dr. Klein and his colleagues pioneered the field of melatonin synthesis, in particular in the pineal gland, the organ of melatonin synthesis in all vertebrates. A distinctive feature of melatonin is its circadian synthesis pattern with high levels during the dark phase and low levels during the light phase. Rhythmicity of melatonin synthesis is achieved by the circadian master clock located in the suprachiasmatic nuclei (SCN) which is under retinal light control. In the present Research Topic, Dr. Klein and colleagues report the latest advances in characterizing the cell types constituting the pineal gland. They defined nine types in the rat pineal gland based on single-cell RNAseq (Coon et al.). Pinealocytes were estimated to account for about 90% of the cells and the remainders are astrocytes, microglia, vascular, and leptomeningeal cells (VLMCs) and endothelial cells. Among the pinealocytes, alpha and beta types are transcriptionally distinct, the beta type representing 95% of all pinealocytes. The authors speculate about a cooperative behavior between both cell types with alpha-pinealocytes being the most specialized and active melatonin synthesizing cells and beta-pinealocytes supporting alpha cells by providing the melatonin precursor N-Acetylserotonin. This kind of single cell analysis is likely to shape our understanding of pineal cell biology in the future.

The issue of melatonin synthesis is then taken further by Dr. Reiter, one of the pioneers in melatonin biology. The authors ask the question of the very first origins of melatonin that is likely to have occurred before the development of the pineal gland. They are advocating for a bacterial origin of melatonin synthesis that is maintained in all plant and animal cells containing mitochondria and chloroplasts, which are considered to represent ancient bacteria originally engulfed by ancient eukaryotic unicells (Zhao et al.). This viewpoint considerably opens our perspective of pineal melatonin in vertebrates and poses the question of the function(s) and putative molecular targets of melatonin in early evolution.

The issue of melatonin targets is taken up by Dr. Jockers and colleagues who summarize and comment on the high number, more than 15, of reported melatonin targets (Liu et al.). Among them those with the highest affinity for melatonin are those found in vertebrates, the G protein-coupled

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MT_1 and MT_2 receptors (1). The affinity of melatonin for these receptors (~0.1 nM) matches physiological circulating melatonin levels of 5 to 150 pg/ml (~0.65 nM). Further receptors, enzymes, pores, transporters, etc., have been suggested to interact with melatonin at higher (up to millimolar) concentrations (Liu et al.). The chemical properties of melatonin itself as a free radical scavenger in *in vitro* assays hint to additional non-receptor mediated properties of melatonin as a cellular antioxidant (Zhao et al.).

By activating MT_1 and MT_2 receptors, melatonin entrains circadian rhythms, regulates seasonal reproduction and retinal physiology, and initiates sleep, the latter discussed in the present Research Topic by Drs. Gobbi and Comai (Gobbi and Comai). Non-selective agonists targeting MT_1 and MT_2 receptors like Ramelteon are already marketed for insomnia. The latest pharmacological and knockout studies in mice indicate that subtype selective agonists might be even a better therapeutic choice as MT_1 and MT_2 receptors have opposing functions on sleep regulation.

Melatonin's metabolic effects are increasingly recognized and summarized here by Dr. Tosini and colleagues, who made important contributions to the field by studying receptor knockout mouse models (Owino et al.). Central melatonin receptors in the SCN might be important metabolic regulators as the dysregulation of the circadian master clock in these brain nuclei contribute to metabolic diseases like type 2 diabetes mellitus (2). Melatonin receptors are also found in peripheral tissues such as the liver, muscle, and pancreas where they participate in glucose homeostasis and body weight regulation. The translation of these animal studies into humans remains to be shown. The association of genetic variants of the *MTNR1B* gene, encoding the MT_2 receptor, with risk of T2DM argues in this direction.

The role of melatonin in reproduction is well established in seasonal breeders. In humans, which are not seasonal, the situation is less clear as summarized by Dr. Olcese, a pioneer in this field. In his article (Olcese), he discusses controversial findings on melatonin and puberty, the entraining role of circulating maternal melatonin on the fetus and its role in oogenesis. Potential therapeutic applications in infertility and melatonin's synergism with oxytocin in inducing labor are also discussed.

Neurodegenerative diseases such as Parkinson and Alzheimer disease represent a challenge for our aging society. Dr. Cardinali, a pioneer of melatonin biology and its clinical translation, advocates in his article for the supplementation of the age-related decline of melatonin levels in the prevention of neurodegenerative diseases (Cardinali). Beneficial effects of melatonin include its hypnotic, chronobiotic, and cytoprotective properties to improve sleep latency and alleviate sundowning, to stabilize biological rhythms and to limit to a low degree inflammatory damage and neuronal death.

The last word of this Research Topic is due to Dr. Arendt, the "Grande Dame" in the melatonin field. By studying free-running blind subjects, she demonstrated the entrainment effect of exogenous melatonin in humans among many other finding (Arendt). Concerning the function of endogenous melatonin, she advocates that melatonin acts as a brake on abrupt short-term changes of circadian phase by maintaining the circadian status quo. Abrupt changes in the light-dark cycle are experienced by shift workers and time zone travelers and the list of associated risks is substantial (increased rate of accidents, lowered alertness and performance, gut problems, increased risk for metabolic diseases, etc.).

In conclusion, the contributions found in the Research Topic on "Melatonin in Health and Disease" illustrate the power and promises—but also the questions and limitations—in the melatonin field as seen by the experts. The future goal for all remains the same: "… to define the known, to evaluate the novel, and ultimately to inspire unknown future research into this fascinating, ancient molecule."

AUTHOR CONTRIBUTIONS

Both authors contributed to the writing. All authors contributed to the article and approved the submitted version.

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We would like to dedicate this editorial to our dear colleague, Franco Fraschini (University of Milano), a pioneer of the melatonin field who passed away recently.

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REVIEW
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Differential Function of Melatonin MT_1 and MT_2 Receptors in REM and NREM Sleep

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The pathophysiological function of the G-protein coupled melatonin MT_1 and MT_2 receptors has not yet been well-clarified. Recent advancements using selective MT_1/MT_2 receptor ligands and MT_1/MT_2 receptor knockout mice have suggested that the activation of the MT_1 receptors are mainly implicated in the regulation of rapid eye movement (REM) sleep, whereas the MT_2 receptors selectively increase non-REM (NREM) sleep. Studies in mutant mice show that MT_1 knockout mice have an increase in NREM sleep and a decrease in REM sleep, while MT2 knockout mice a decrease in NREM sleep. The localization of MT_1 receptors is also distinct from MT2 receptors; for example, MT_2 receptors are located in the reticular thalamus (NREM area), while the MT_1 receptors in the Locus Coeruleus and lateral hypothalamus (REM areas). Altogether, these findings suggest that these two receptors not only have a very specialized function in sleep, but that they may also modulate opposing effects. These data also suggest that mixed MT_1-MT_2 receptors ligands are not clinically recommended given their opposite roles in physiological functions, confirmed by the modest effects of melatonin or MT_1/MT_2 non-selective agonists when used in both preclinical and clinical studies as hypnotic drugs. In sum, MT_1 and MT_2 receptors have specific roles in the modulation of sleep, and consequently, selective ligands with agonist, antagonist, or partial agonist properties could have therapeutic potential for sleep; while the MT_2 agonists or partial agonists might be indicated for NREM-related sleep and/or anxiety disorders, the MT_1 agonists or partial agonists might be so for REM-related sleep disorders. Furthermore, MT_1 but not MT_2 receptors seem involved in the regulation of the circadian rhythm. Future research will help further develop MT_1 and/or MT_2 receptors as targets for neuropsychopharmacology drug development.

Keywords: melatonin, MT_1 receptor, MT_2 receptor, sleep, REM, NREM

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SLEEP, SLEEP ARCHITECTURE, AND SLEEP DISORDERS

Following Tononi and Cirelli's synaptic homeostasis hypothesis (1), sleep is the price the brain pays for plasticity. Indeed, during waking, the learning process requires the strengthening of connections throughout the brain. This process increases cellular need for energy and supplies, decreases signal-to-noise ratios, and saturates learning. During sleep, cerebral spontaneous activity

renormalizes the net synaptic strength and restores cellular homeostasis. This activity of synapses during sleep may also explain the benefits of sleep on memory acquisition, consolidation, and integration (1).

In mammals, physiological sleep is comprised of two distinct states called rapid-eye movement (REM) sleep and non-REM (NREM) sleep that alternate through the night in a cyclical fashion. REM occurs in short periods, characterized by a decrease in muscle tone and associated with a profound sympathetic activation, including increased heart rate, breathing, blood pressure, and temperature. NREM periods are longer and are associated with a parasympathetic activation, consisting of low blood pressure, low heart rate, and decreased temperature. While structured dreams occur mostly in REM, non-structured and bizarre dreams occur in NREM. In adults, about 75–80 percent of total time spent in sleep is spent in NREM sleep while the remaining 20–25 percent occurs in REM sleep. During the night, adult subjects usually experience four to five NREM to REM sleep cycles. Interestingly, newborns spend more time in REM, and the time spent in NREM increases progressively over the years at the expense of REM.

NREM sleep is divided into progressively deeper stages—named stage N1, stage N2, and stage N3—that can be distinguished based on specific electroencephalogram (EEG) traits [for details on this topic, which is beyond the aim of this review, please see Atkin et al. (2) and Iber et al. (3)]. However, it is important to highlight that stage N3, commonly referred to as slow wave sleep (SWS) during which there is deep or delta-wave sleep, seems important for cerebral restoration and recovery, the maintenance and consolidation of memory (4), and metabolic regulation (5). As a consequence, disturbances in the duration and architecture of sleep is often associated with next-day impairments in conducting daily activities and, if not treated, can be closely linked to many neurological and psychiatric disorders (6–8). The lack or the disruption of sleep, known as "insomnia," is a common public health problem, with a prevalence ranging from 11 to 16% (9).

The publication of the 5th edition of the *Diagnostic and statistical manual of mental disorders* (DSM-V) (10) fundamentally changed the landscape of sleep medicine and the diagnosis of insomnia. The DSM-IV distinguished primary insomnia [characterized by a difficulty to initiate or maintain sleep for at least 1 month, with associated daytime fatigue, significant distress or social impairment (9, 10)] from insomnia secondary to another diagnosis (including major depressive disorder and generalized anxiety disorder). Instead, the DSM-V has eliminated primary insomnia as a diagnosis in favor of "insomnia disorder," which may occur alongside other diagnoses like major depressive disorder. This revised definition obliges the clinician to treat insomnia as a distinct mental condition, even if it may be present with other mental disorders (2).

Insomnia is frequent in people suffering from major depression, with alterations in sleep neurophysiology, notably decreased SWS, reduced REM latency and increased REM density. Increased REM density has also been observed in eating disorders, narcolepsy, presenile dementia, and other neuropsychiatric diseases (11).

Besides "insomnia disorder," mostly characterized by a decrease in NREM quantity and longer latency to sleep (first episode of NREM), the DSM-V, like DSM-IV, proposes a specific classification for REM sleep behavior disorders. REM sleep behavior disorders are characterized by recurrent episodes of arousal during sleep associated with vocalization and/or complex motor behaviors that arise during rapid eye movement (REM) sleep, confusion or disorientation on waking from these episodes, co-presence of REM sleep without atonia on polysomnographic recordings, and/or history of synucleinopathy diagnosis (e.g., Parkinson's disease, multiple system atrophy).

From a pharmacological point of view, it is thus important that a drug used to treat insomnia or sleep-related disorders not only acts on the duration of sleep but also preserves the physiological sleep architecture. Unfortunately, most of the currently available hypnotics considerably alter the physiological sleep architecture (2). In addition, official medicine has not yet recognized guidelines for specific treatment of "NREM disorders" vs. "REM disorders," and hypnotics are non-differentially used for both conditions.

MELATONIN AND SLEEP: PRECLINICAL AND CLINICAL FINDINGS

Currently used hypnotic drugs, such as benzodiazepines and thier derivates (i.e., zopiclone), act mostly on the GABAergic system, increasing SWS and decreasing REM sleep, thus altering the sleep architecture (2). This can result in next-day cognitive impairments and may also lead to abuse. Antidepressants, such as tricyclics and selective serotonin reuptake inhibitors (SSRIs) mostly reduce REM density, with little or no effect on SWS. The catecholamine releaser bupropion increases REM and has no effect on SWS (2, 12, 13). To develop new effective hypnotic drugs that selectively increase SWS without altering REM density and the whole sleep architecture therefore remains a scientific and medical challenge.

The physiological effects of melatonin (N-acetyl-5-methoxytryptamine, MLT) in the brain result from the activation of high-affinity (Ki $\approx$ 0.1 nM), G protein-coupled receptors, referred to as MT_1 and MT_2. Activation of both receptors mainly activates G_i proteins with inhibition of adenylyl cyclase and subsequent decrease of intracellular cAMP levels. Detailed information on the molecular signaling pathways activated by melatonin receptors is beyond the scope of the aim of the present work and can be found in the reviews by Dubocovich et al. (14), Jockers et al. (15), and Oishi et al. (16). However, of interest, recent lines of research have indicated that melatonin receptors can form abundant MT_1/MT_2 hetero-oligomers and that they can both heteromerize with other receptors, including the serotonin 5-HT_{2C} (17). Importantly, from both neurobiological and pharmacological perspectives, these heteromers display functional properties different from those of the corresponding homomers (17). For example, in the MT_2/5-HT_{2C} heteromer, melatonin binding induces the activation of G_q signaling through a transactivation of the serotonergic receptor caused

by conformational changes of the MT_2, which is normally not coupled to a G_q (17).

Due to the lack of selective ligands for MT_1 and MT_2 receptors, the respective roles of these receptors in brain function and in particular in sleep regulation remain unclear.

The neuromodulator MLT is synthesized by the pineal gland and has been reported to have hypnotic effects on humans, although these results are still controversial (18–21). Meta-analysis on the effects of melatonin indeed suggest that melatonin has a soporific effect, helping people to fall asleep, but has no effects on sleep maintenance and sleep quality (18, 19).

Similarly, in laboratory animals, several studies have demonstrated that MLT reduces time to sleep onset and increases SWS and REM (22–24), effects that would be blocked by the $GABA_A$ receptor antagonists flumazenil and picrotoxin (24). Others have suggested that MLT regulates REM, since lesioning of the pineal gland or the inhibition of MLT synthesis reduce REM density during light and dark periods (25–27). The effects of MLT (3–5 mg/kg) in Djungarian hamsters and rats (both nocturnal animals) were short lasting and depended on the time of day. MTL prolonged sleep latency in the late light period, enhanced sleep fragmentation in the early light period, and elevated body temperature. REM sleep was reduced when hamsters were treated with MLT after the late light period and when rats were treated after dark onset. These indicate that MLT induces changes that are typical for the dark period of each species, i.e., wakefulness in the nocturnal Djungarian hamster and rat, and sleepiness in diurnal animals (28).

Electrophysiological recordings in monkeys have indicated that MLT has only a weak and transient effect on sleep in these species (29, 30), decreasing the latency of the first episode of sleep (31).

Five non-selective MT_1/MT_2 agonists—ramelteon (S)-*N*-[2-(1,6,7,8-tetrahydro-2*H*-indeno[5,4-*b*]furan-8-yl)ethyl] propionamide, tasimelteon (VEC-162; structure not disclosed), TIK-301 (β-methyl-6-chloroMLT; *N*-[(2R)-2-(6-chloro-5-methoxy-1*H*-indol-3-yl)propyl]acetamide), agomelatine (N-[2-(7-methoxynaphthalen-1-yl)ethyl]acetamide) and piromelatine (N-[2-(5-methoxy-1H-indol-3-yl)ethyl]-4-oxopyran-2-carboxamide—have been tested in different species for potential use in insomnia. Ramelteon seems to have insignificant effects on sleep in rats (32), monkeys (31), and cats (29). Agomelatine, on top of being a non-selective MT_1/MT_2 agonist, also acts as an antagonist at the level of $5\text{-}HT_{2C}$ receptors. Agomelatine increases NREM and REM sleep in rats but only if administered shortly before the dark phase (active phase for rodents), but not during the light phase (inactive phase for rodents) (32). In the same experiment, melatonin increased REM sleep, which was followed by an increase in wakefulness (32). Tobler et al. (30) found that melatonin and agomelatine (S-20098) reduced the power density in NREM sleep in the low frequency range (1–8 Hz), but did not affect the vigilance states and brain temperature. Sleep data with tasimelteon and TIK-301 in rats are lacking (33).

A summary with the preclinical data investigating the effects of melatonin and non-selective MT_1/MT_2 agonists on the sleep/wake cycle of rats is reported in **Table 1**.

Ramelteon (37–39), tasimelteon (40), and TIK (41, 42) have also been tested in humans for the treatment of insomnia. All three significantly reduced the latency to sleep in humans, but their effect on total sleep time was minimal.

In particular, the non-selective MT_1-MT_2 receptor ramelteon decreases the latency of sleep but not the whole duration (39, 43) and for this reason was approved by the Food and Drug Administration (FDA, United States) but not the European Medicines Evaluation Agency (EMEA) because "*... the difference in the time taken to fall asleep between patients taking Ramelteon and those taking placebo was considered to be too small..... When other aspects of sleep were considered, Ramelteon did not have any effect*." (https://www.ema.europa.eu/medicines/human/withdrawn-applications/ramelteon, consulted on November 1, 2018).

Similarly, the non-selective agonist tasimelteon (VEC-162) was effective for treatment of transient insomnia associated with shifted sleep and wake time (44) and was developed as an orphan drug for the treatment of Non-24-H Sleep-Wake Disorder, but not for insomnia. The EMEA approved tasimelteon for the same condition but only in completely blind people (https://www.ema.europa.eu/documents/assessment-report/hetlioz-epar-public-assessment-report_en.pdf, consulted on November 1, 2018).

Agomelatine was also approved by the EMEA as an antidepressant, but a recent meta-analysis has pointed out its low effects compared to other classes of antidepressants (45), some clinical evidence has shown that agomelatine could be efficacious in sleep disorder (46), especially if associated with depression (47).

Piromelatine is a $MT_1$and MT_2 agonist with agonism also at $5\text{-}HT_{1A/1D}$ receptors (48). Piromelatine was shown to have both hypnotic and antinociceptive effects by electroencephalogram (EEG) recordings in an animal model of neuropathic pain, partial sciatic nerve ligation (PSL) (49). It increases NREM sleep and decreases wakefulness in PSL mice, but the effect could be blocked by preadministration of a melatonin receptor antagonist, a $5\text{-}HT_{1A}$ receptor antagonist, or an opiate receptor antagonist (49), demonstrating a lack of selectivity for the melatonin receptors.

In 2013, Neurim Pharmaceuticals Ltd announced positive results from a phase II randomized clinical trial ($N = 120$) of piromelatine for the treatment of primary insomnia (50). Active treatment with piromelatine at 20 or 50 mg/d over 4 weeks resulted in significantly improved wake after sleep onset (WASO). However, the primary outcome of latency to persistent sleep was not significant when compared with the placebo (https://clinicaltrials.gov/ct2/show/results/NCT01489969) and consequentiality, the company did not further develop piromelatine for insomnia. The Clinicaltrials.gov database lists a study currently recruiting patients entitled "Safety and Efficacy of Piromelatine in Mild Alzheimer's Disease Patients (ReCOGNITION)," https://clinicaltrials.gov/ct2/show/NCT02615002, indicating that the compound will be primarily

TABLE 1 | Acute effects of melatonin, non-selective MT_1/MT_2 receptors agonists, and selective MT2 receptors partial agonists, agonists and antagonists on sleep/wake stages of rats during the 24-h light/dark cycle.

	Latency to NREM sleep	NREM sleep duration	REM sleep duration	Wakefulness duration
Melatonin	ø (32) ↓ (23) n.r. (30)	ø (23, 30) Dark phase: ↓↑ depending on time after administration (32) Light phase: ø (32)	ø (23, 30) Dark phase:↓↑ depending on time after administration (32) Light phase: ø (32)	ø (23, 30) Dark phase: ↑ depending on time after administration (32) Light phase: ø (32)
Non-selective MT_1/MT_2 receptors agonist UCM793 (34)	ø	ø	ø	ø
Non-selective MT_1/MT_2 receptors agonist Agomelatine	n.r. (30) ø (32)	ø (30) ↑ Dark Phase (32) ø light phase (32)	ø (30) ↑ Dark Phase (32) ø light phase (32)	ø (30) ↓ Dark Phase (32) ø light phase (32)
Non-selective MT_1/MT_2 receptors agonist Ramelteon	↓ (35) ø (32)	↑ (35) Dark phase: transient ↑ 4 h after administration (32) Light phase: ø (32)	ø (35) Dark phase: transient ↑ 4 h after administration (32) Light phase: ø (32)	↓ (35) Dark phase: transient ↑ 4 h after administration (32) Light phase: ø (32)
Selective MT_2 receptors partial agonists UCM765 (34) and UCM924 (23)	↓	↑	ø	↓
Selective MT_2 receptors agonist IIK7 (36)	↓	↑	ø	n.r.
Selective MT_2 receptors antagonist 4P-PDOT (34)	ø	ø	ø	ø

↓, decrease; ↑, increase; ø, no change; n.r., not reported.

developed for cognition and not for sleep (a secondary outcome of the study).

Altogether, these animal and clinical studies have pointed out the equivocal effects of non-selective agonists on sleep duration, despite the undoubted evidence that MLT receptors are implicated in sleep regulation and circadian rhythms.

MT_1 AND MT_2 RECEPTORS AND SLEEP

Sleep is regulated by two processes, the sleep/wake homeostasis and the circadian clock (51). In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN receives direct inputs about the external environmental day/night cycle from the retina via the retino-hypothalamic tract, and then accordingly, controls the synthesis of melatonin by the pineal gland. In turn, melatonin controls the SCN activity via a feedback mechanism involving MT_1 and MT_2 receptors located in the SCN (52).

Insights From Pharmacological Studies With MT_2 Selective Ligands

In our lab, we used electroencephalogram (EEG) and electromyogram (EMG) recordings in rats for 24 h to examine the effects of the selective MT_2 partial agonists UCM765 and UCM924 on sleep in comparison with diazepam, melatonin and the non-selective MT_1-MT_2 agonist UCM793 (23, 34).

We observed that UCM765 decreased the latency to the first episode of NREM sleep and increased the total amount of NREM sleep, in particular during the light (non-active) phase. We then compared the effects of UCM765 with those of the clinically-used hypnotic drug diazepam, and observed similar effects on both latency to the first episode and duration of NREM sleep. However, we found that, unlike diazepam, UCM765 did not induce a significant suppression of delta power activity during NREM sleep (34). Similar to melatonin, the MT_1/MT_2 non-selective agonist UCM793 did not produce significant effects on sleep stages (34), suggesting that the MT_2 receptor subtype is probably the one mainly involved in the regulation of NREM sleep but the MT1 may counterbalance the MT_2-mediated effects. This hypothesis is also supported by the fact that knockout mice for both MT_1 and MT_2 receptors (53), as well as pinealectomized rats (54), do not show impairments of NREM and REM sleep duration. However, we cannot exclude the possibility that melatonin acts on sleep through mechanisms independent of MT_1/MT_2 activation. Indeed, unlike the non-selective MT_1/MT_2 agonist UCM793 (34), melatonin significantly reduced the latency to NREM sleep onset but not to REM sleep onset (23). Evidence has shown that melatonin can interact with other neurotransmitter systems implicated in the neurobiology of sleep (2) including alpha-7 nicotinic (55, 56) or GABA (24) receptors and the release of serotonin (57, 58).

Targeting the MT_2 receptors with the MT_2 agonist IIK7 also selectively increased the duration of NREM sleep without affecting REM sleep, although the overall effects appear to be only transient (36). Future studies should investigate possible differential effects on NREM sleep produced by partial and

full agonists toward the MT_2 receptors. There is not yet a clear understanding of whether and how MT_1 and MT_2 receptors may desensitize upon stimulation by exogenous melatonin/selective ligands and according to the daily fluctuating levels of endogenous melatonin. Nonetheless, it appears that the hypnotic effects of MT_2 partial agonists may be superior to those of MT_2 full agonists, because the former would avoid the rapid desensitization induced by the full agonist. The higher pharmacological efficacy of MT_2 partial agonists over melatonin has also been found when comparing their analgesic effects in preclinical models of neuropathic pain (59, 60).

Given the paucity of selective MT_1 receptor ligands, only one pharmacological study has explored the effects of MT_1 receptor activation/inhibition upon the sleep stages, indicating a possible selective effect of MT_1 receptor selective ligands on REM sleep activation (61).

Validating MT_2 Receptors as a Target to Selectively Promote NREM Sleep

UCM765 and UCM924 have shown high selectivity and affinity toward MT_2 receptors and low affinity toward a panel of many other receptors (59) known to be involved in sleep (2, 62). Furthermore, the role of MT_2 receptors in the observed effects of these two drugs on sleep has been validated using both pharmacological and genetic approaches. The MT_2 receptor antagonist cis-4-phenyl-2-propionamidotetralin (4P-PDOT) is a reference compound exhibiting good binding affinity for the human cloned MT_2 receptor ($pK_i = 8.8$), and a selectivity for the MT_2 receptor at least 100 fold that of the MT1 subtype (63). In order to test the hypothesis that the promotion of NREM sleep is MT_2-mediated, we administered 4P-PDOT (10 mg/kg, a dose not affecting sleep stages) 10 min prior to UCM765, and found that 4P-PDOT completely blocked the effects of UCM765 on NREM sleep duration (34). UCM765 was also tested in MT_2KO, and, unlike in wild-type control mice, the compound did not enhance NREM sleep in the MT_2KO animals. These data strongly confirm the important role of MT_2 receptors in modulating NREM sleep.

Insights From MT_1, MT_2 and Double MT_1-MT_2 Receptors Knockout Mice

The role of melatonin receptors in sleep has also been investigated by taking advantage of knockout mice for MT_1 and/or MT_2 receptors.

Quite surprisingly, the lack of both MT_1 and MT_2 receptors did not significantly affect the amount of NREM and REM sleep during the 24 h (53). In contrast, a slight but significant increase in the time of wakefulness during the 24 h was present (53). These findings suggest that the lack of both melatonin receptors only minimally influences the two sleep stages, in agreement with the finding that also the lack of melatonin (their physiological ligand) due to a pinealectomy does not significantly affect the duration of sleep (54).

In keeping with the pharmacological studies reported above, the genetic inactivation of only one of the two melatonin receptor subtypes instead produces significant effects on the sleep stages. MT_2KO mice display a significant reduction of NREM sleep duration during 24 h, with the decrease mainly due to an effect occurring during the light (inactive) phase (34, 53). No effects on REM sleep duration have been observed in MT_2KO mice (34, 53). These findings in MT_2KO mice corroborate pharmacological findings with MT_2 agonists/partial agonists (23, 34, 36) demonstrating a selective role of MT_2 receptors in regulating NREM sleep.

In MT_1KO mice a significant decrease in the duration of REM sleep has been observed (34, 53), suggesting a central role for MT_1 receptors in REM sleep regulation. In contrast, the possible involvement of MT_1 receptors in NREM sleep remains unclear. While in rats there is concordance among studies on how to score sleep stages, different protocols have been used in mice. In particular, sleep is scored using either 4 or 10 s epochs (64). However, given that in mice very short episodes of REM sleep are present, the 4 s epoch seems probably the best way to score sleep in mice (64). In keeping with this rationale, we found that the duration of NREM and REM sleep in MT_1 mice can slightly differ depending on the 4 or 10 s methodological approach. Using 4 s epochs, we found no change in NREMS in MT_1KO mice compared with WT control animals. In contrast, using 10 s epochs we found a slight but significant increase of NREM sleep during the dark/active phase in MT_1KO mice compared with WT. These different results with 4 and 10 s analyses suggest a disruption of microarchitecture of REM in MT_1KO; moreover, the opposing effects in NREM detectable with the 10 s analyses point out the opposing effects of MT_1 and MT_2 on NREM sleep: while MT_1KO have an increase in NREM, the MT_2KO have a decrease.

Interestingly, MT_1KO also show an impairment at the level of dark-light cycle of the REM sleep: the quantity of REM is the same in the dark and light periods, suggesting the involvement of this receptor also in the circadian regulation of REM sleep.

Collectively, as summarized in **Table 2**, the study of the 24-h sleep/wake cycle in melatonin receptors knockout mice indicates that MT_1 receptors are mostly involved in REM sleep regulation while MT_2 receptors in NREM sleep.

Localization of Melatonin Receptors in Brain Regions Involved in Sleep Regulation

The SCN is the pacemaker of the circadian rhythms in the body, including the sleep-wake cycle. Both MT_1 and MT_2 receptors have been reported at the level of the SCN; however, while

TABLE 2 | 24-h sleep/wake stages in MT_1KO, MT_2KO, and MT_1/MT_2KO mice.

	NREM sleep duration	REM sleep duration	Wakefulness duration
MT_1KO	ø (4 s epochs) ↑ (10 s epochs)	↓	ø (4 s epochs) ↓ (10 s epochs)
MT_2KO	↓	ø	↑
MT_1/MT_2KO	ø	ø	↑

Data obtained from Comai et al. (53). ↓, decrease; ↑, increase; ø, no change; by scoring sleep/wake stages using both 4 and 10 s epochs unless otherwise specified.

the presence of MT_1 receptors has been demonstrated with several techniques such as RT-PCR, *in-situ* hybridization and immunohistochemistry (65–67), the data on the presence of MT_2 receptors are not yet so clear and points only to a very low expression (65, 67, 68). Our laboratory has shown that MT_2 receptors are located in critical areas for sleep functions. From rostral to caudal, strong, selective MT_2 immunoreactivity of neuronal cell bodies and proximal dendrites was consistently observed in key brain regions: the septum, CA_2 layers of the hippocampus, supraoptic nucleus, reticular nucleus of the thalamus, red nucleus, substantia nigra pars reticulata, oculomotor nuclei, and ventral tegmental nucleus (65). Moderate MT_2 immunoreactivity was also seen in the ventral pallidum, internal globus pallidus, other sectors of the hippocampus (e.g., the dentate gyrus), paraventricular nucleus of the hypothalamus and inferior colliculus (65).

The reticular thalamus (RT) is a small area whose activation promotes NREM sleep by connecting deeper brain structures to cortex via thalamo-cortical pathways. RT generates the classic silent/burst rhythmic activity during episodes of NREM sleep (69–71). During episodes of NREM sleep, RT neurons discharge in a slow, rhythmic, burst-firing mode that is transmitted to thalamic relay nuclei and modulated by corticothalamic inputs, resulting in a widespread synchronization across neuronal assemblies (72, 73). In rats, the selective MT_2 receptor partial agonist UCM765 induces at the level of RT neurons a rhythmic synchronized burst activity separated by periods of silence, characterized by an increased percentage of spikes in burst, an increase in mean spike per burst and a decrease in mean interburst time (34). Since this rhythmic activity promotes NREM sleep, MT_2 receptors may thus be viewed as a key component in sleep regulation. Of note, the activation of RT neurons by UCM765 is MT_2 receptor-mediated, since the local infusion of 4P-PDOT blocked the effects of the drug upon the neurons, and is sufficient to promote NREM sleep. Indeed, when UCM765 is injected in a brain region not primarily involved in sleep regulation but containing MT_2 receptors such as the substantia nigra pars reticulate, no effects on NREM sleep has been observed (34).

Recently, Sharma et al. (74) found in mice that orexin neurons in the perifornical lateral hypothalamus (PFH) express MT_1 but not MT_2 receptors. Orexins, also known as hypocretins, are neuropeptides synthesized in the brain exclusively by neurons in the lateral hypothalamic area that makes excitatory connections to all of the arousal-promoting nuclei. Orexins are thus a crucial neurotransmitter in promoting wakefulness, and indeed melatonin injected at the level of PFH was able to induce sleep (74). Following this finding, Sharma et al. (74) claimed that melatonin via MT_1 receptors in the PFH may induce sleep. It is our opinion that this claim requires further proof-of-concept studies (75), but MT_1 receptors present in the PFH are likely to contribute to effects of melatonin upon the sleep-wake cycle.

We also found MT_1 receptors at the level of 5-HT neurons in the dorsal raphe (65), and the lack of MT_1 receptors in MT_1KO mice impaired the physiological light-dark fluctuation of a subpopulation of dorsal raphe 5-HT neurons (76). Monoaminergic neurons fire at a steady rate during wakefulness, decrease their firing during NREM sleep, and are virtually silent during REM sleep (2). Future studies are thus warranted to examine whether MT_1 receptors present on 5-HT neurons are involved in the modulation of sleep.

MT_1 and MT_2 Receptors and Sleep Circuits: Possible Interactions

It is important at this point to improve our understanding of how the MT_1 and MT_2 receptors play their roles in the complex sleep circuitry composed of different brain nuclei and receptors.

The neural circuits that generate arousal and sleep (both NREM and REM) remain to be completely elucidated.

Humans are diurnal mammals, with a circadian clock that promotes wakefulness during the day. Sleep timing is phase-linked to intrinsic circadian rhythm-controlled temperature rhythms as well as extrinsic light and dark signaling (77). Homeostasis is another sleep regulator, meaning that the decrease of sleep for one night induces an increase in deep sleep quantity and quality the following night.

The manner in which the brain alternates cycles of NREM and REM remains unknown; however, a prominent role for melatonin receptors can be hypothesized. The melatonin receptors MT_1 and MT_2 are both present at the level of retina, but MT_2 mRNA seems to be absent in retinal ganglion cells (78). The retinohypothalamic tract, which contains the intrinsically photosensitive retinal ganglion cells (ipRGC) and the photopigment melanopsin, inputs directly and monosynaptically to the SCN, an area rich in MT_1 and MT_2. Circadian signals from the SCN are transmitted sequentially to the paraventricular nuclei (PVN), intermediolateral nucleus of the spinal cord (IML), superior cervical ganglion (SCG), and finally the pineal gland (79). Bilateral SCN lesion abolishes circadian rhythms of melatonin synthesis and secretion, demonstrating that the SCN is the melatonin rhythm generator (80). The pineal gland produces melatonin when stimulated by the SCN glutamatergic neurons (in response to the darkness) (79). MLT is then released into the bloodstream through which it reaches every organ in the body, including the brain where it interacts with MT_1 and MT_2 located in the NREM areas (including RT) or REM area [including locus coeruleus (LC) and lateral hypothalamus (LH)]. These areas regulate in concert the different sleep cycling. It may be hypothesized that the peak of melatonin between 12 and 3 a.m. may desensitize or down-regulate its own receptors, generating a differential expression and/or sensitivity of MT_1 (REM sleep) and MT_2 (NREM sleep) that may in their turn generate a kind of rhythmic balance between NREM, REM and wakefulness. In support of this theory, it has been shown that MT_2 receptors desensitize quickly after melatonin exposure (81).

Melatonin stimulates the brain's MT_2 receptors in the NREM sleep-activating regions of the brain: the reticular thalamus and the preoptic areas, including both the ventrolateral preoptic area (vlPO) and the median preoptic area (MNPO) (34, 65). Specifically, the MNPO appears to regulate the firing activity of the vlPO (82). During the transition from wakefulness to sleep, the MNPO—which specifically contains neurons that fire

during SWS and paradoxical or REM sleep, with slow discharging activity <5 Hz—begin to fire not before, but after, sleep onset, with a gradual increase in discharge rate (83).

During NREM sleep, two nuclei are particularly active: the RT, containing melatonin MT_2 and GABA receptors and responsible for thalamocortical input to the prefrontal cortex (showing synchronized activity during NREM); and the ventrolateral preoptic area (vlPAG), containing GABA and galanin receptors, and inhibiting noradrenergic, serotonergic, cholinergic, histaminergic, and hypocretinergic neurons. These nuclei play a role in the "reciprocal inhibitory" model of the sleep–wake switch. In particular, during NREM sleep, the vlPO sends inputs that reduce the activity of the orexinergic arousal system and the monoamine nuclei [including the Ventral tegmental area (VTA) containing dopamine (DA) neurons, the dorsal raphe (DR) containing serotonin (5-HT) neurons, and the LC containing norepinephrine (NE) neurons] by releasing the inhibitory neurotransmitters GABA and galanin. As a feedback mechanism, vlPO neurons receive reciprocal inputs from the arousal nuclei including the VTA, DR, and LC; the vlPO also receives input from the histaminergic tuberomammillary nucleus (TMN) (84).

People suffering from fatal familial insomnia (FFI) show thalamic disruption that inactivates their ability to sleep, which is paralleled by a dysfunction in melatonin production (85). As mentioned before, the RT neurons discharge in burst activity exclusively during NREM, and thalamocortical pathways project this synchronous burst activity, intermingled with periods of silence, onto the cortex. This rhythmic firing activity generates the synchronized EEG pattern typical of SWS, which produces disconnection between the cortex and the outside world (86). Remarkably, the RT is also rich in melatonin MT_2 receptors, which are likely activated at the beginning of NREM sleep (34). These receptors, which are contribute to the generation of the characteristic bursts that, through the thalamo-cortical pathways, produce the classical silent/burst activity in the PFC. Conversely, during wakefulness, the RT and thalamocortical neurons are depolarized by inputs from the reticular activating system of the brainstem, and discharge instead with a tonic activity [adapted from Purves et al. (87)].

On the other hand, REM sleep is regulated by other brain areas. The vlPAG is a putative "REM ON" nucleus, switching the brain to the REM sleep mode. During REM, the sublateral nucleus (SLD), the basal forebrain (BF), and the lateral tegmentum/ pedunculopontine tegmentum (LDT/PPT, rich in acetylcholine receptors) and the ventromedial medulla (VM) neurons become particularly active.

Many researchers have hypothesized that REM sleep is mediated mostly through cholinergic neurons located in the LDT/PPT. These neurons are active during REM sleep and generate the cortical activation and atonia typical of this sleep stage, and are inactive during NREM sleep. Indeed, LDT/PPT neurons send inputs to the ventromedial medulla (VM), which inhibits motor neurons by releasing GABA and glycine into the spinal and brainstem motor neurons, producing atonia. LDT/PPT neurons are also the main source of acetylcholine (Ach) to the thalamus: activation of this ACh pathway depolarizes thalamic neurons, generating the cortical activation associated with REM sleep and dreaming. Other nuclei important for REM sleep regulation are: (1) the sublaterodorsal nucleus (SDL) which produces GABA and glutamate and projects to the glycinergic/GABAergic premotor neurons in the ventromedial medulla and ventral horn of the spinal cord, and through these circuits likely inhibits motor neurons during REM sleep; (2) the melanin-concentrating hormone (MCH)-containing neurons that fire during REM sleep and decrease their activity during NREM sleep and wakefulness [Saper et al. (88); reviewed in España and Scammell (62)]; and (3) LC neurons that fire as a function of vigilance and arousal displaying a firing of 4–6 Hz during quiet wakefulness and a sustained activation during alertness or stress. LC NE firing decreases markedly during NREM and is completely silent during REM sleep (89, 90).

Interestingly, we found that the daily circadian changes of LC NE neural activity are blunted in MT_1KO mice as compared with WT controls, and the bust-firing activity of LC NE neurons, that is associated with the synaptic release of the neurotransmitter (91), is significantly reduced in MT_1KO compared with WT mice (76).

Another cholinergic nuclei that is active during REM sleep and wakefulness is the LH which contains both MT_1 and orexin receptors (74).

However, more research, especially with selective compounds or optogenetic techniques, is required to better differentiate the role of these two receptors in sleep regulation. **Figure 1** illustrates the main areas of the brain implicated in the regulation of sleep and wakefulness with their respective receptors, including MT_1 and MT_2.

Melatonin Pick, Circadian Rhythms and MT_1/MT_2 Receptors

The plethora of studies here reported demonstrating the weak hypnotic properties of exogenous melatonin and the fact that melatonin picks in both nocturnal and diurnal animals at the same time—between 1 and 3 a.m.—(92, 93) leads us to hypothesize that melatonin is not *per se* a neuromodulator acting on sleep, but rather a pace-maker influencing circadian rhythms among which the circadian regulation of sleep in both diurnal and nocturnal animals. Melatonin likely acts as an "orchestra conductor": when melatonin peaks (1–3 a.m.) it regulates the expression of MT_1, MT_2, and other non-melatonin receptors, which are those directly regulating sleep stages. On one hand, the nocturnal overexpression of MT_2 receptors in diurnal mammalian increases the propensity to sleep by activating the neurons that trigger NREM sleep (i.e., neurons in the RT). On the other hand, in nocturnal animals, the melatonin peak would down-regulate MT_2 receptors while up-regulating MT_1 and other receptors involved in wakefulness, for example monoamines (76) and orexin (74) receptors.

In support of this hypothesis, Pinato et al. (94) found that in the diurnal primate *Sapajus apella*, MT_1 and MT_2 receptors displayed different reciprocal patterns of expression according to the light/dark cycle in four hypothalamic nuclei, with an apparent inverse expression in the SCN compared with the

other three hypothalamic areas. Pinealectomized rats (54) or humans with pineal parenchymal tumors (95) that display significantly altered rhythms in circulating levels of melatonin do not necessarily show sleep impairments, but in contrast, the activation of MT_2 receptors or the genetic deletion of either MT_1 or MT_2 receptors induces significant changes in sleep stages. In line, the non-selective MT_1-MT_2 agonist tasimelteon, which has been approved for the treatment of non-24-h sleep–wake rhythm disorder in blind people display pharmacological efficacy as a consequence of the resynchronization to a 24-h sleep-wake rhythm (96). Interestingly, this kind of hormonal circadian regulation of the receptors has also been observed for the cortisol peak (occurring early in the morning) and the response of its glucocorticoid and mineralocorticoid receptors (97).

Importantly, similar to cortisol, circulating melatonin may not only play a role in regulating the activity and expression may of its two receptors, but also the expression (98) of clock genes, which in turn regulate a plethora of different cellular functions.

The data reported in this review indicate that the MT_2 receptor is mostly involved in sleep, and less in the regulation of circadian rhythms. In contrast, several studies suggest that the MT_1 receptor is mostly involved in the circadian regulation of behavior.

Indeed, *in-vitro* experiments using SCN slides showed that MT_1 receptors control the neuronal firing rate and MT_2 receptors the phase shift-circadian rhythm of the neuronal firing (52); however, in *in-vivo* studies, a MLT injection phase shifted the SCN activity onset of WT but not of MT_1KO mice and also accelerated the entertainment to a new light-dark cycle of WT

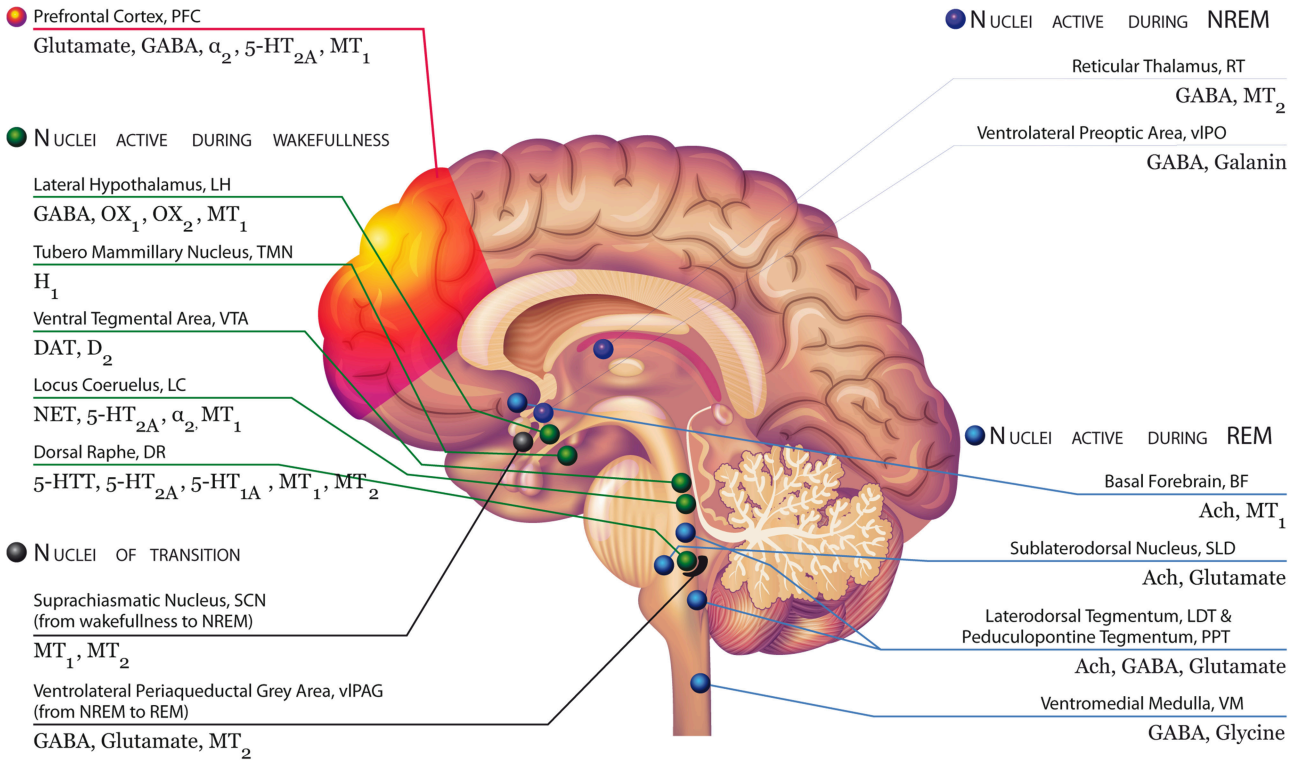

FIGURE 1 | Brain areas involved in the regulation of sleep and wakefulness with their respective receptors, including MT_1 and MT_2 receptors Modified with permission from Atkin et al. (2). **Top left, green:** During NREM, the serotonin neurons of the Dorsal Raphe (DR), the dopaminergic neurons of the Ventral tegmental area (VTA), and the noradrenergic neurons of the Locus Coeruleus (LC) decrease their firing activity. These neurons are silent during REM. OX_1 and OX_2-containing orexinergic neurons of the Lateral Hypothalamus (LH) decrease their firing activity during NREM and REM. The histaminergic H_1-containing neurons of the Tuberomammillary Nucleus (TMN) decrease their firing activity during sleep. During wakefulness, neurons of the arousal system (i.e., monoaminergic neurons, orexinergic neurons) send widespread ascending projections to the cerebral cortex, stimulating cortical desynchronization with high frequency gamma and low frequency theta rhythmic activity. **Bottom left, black:** MT_1 and MT_2 receptors expressed in suprachiasmatic neurons, which receive inputs directly from the retinohypothalamic tract (RHT), influenced by light and external stimuli may be likely involved in the switch from wakefulness to NREM sleep. The transition from NREM and REM is controlled by the ventrolateral periaqueductal gray area (vlPAG), containing GABA, glutamate receptors, but also melatonin MT_2 receptors. **Top right, red:** During NREM sleep, two nuclei are particularly active: the reticular thalamus (RT), containing melatonin MT_2 and GABA receptors, which is responsible for thalamocortical input to the prefrontal cortex (showing synchronized activity during NREM); and the ventrolateral preoptic area (vlPAG), containing GABA and galanin receptors. They inhibit noradrenergic, serotonergic, cholinergic, histaminergic, and hypocretinergic neurons. These nuclei play a role in the "reciprocal inhibitory" model of the sleep–wake switch. **Bottom right, blue:** The vlPAG is a putative "REM ON" nucleus, switching the brain to the REM sleep mode. During REM, the sublateral nucleus (SLD), the basal forebrain (BF), and the lateral tegmentum/ pedunculopontine tegmentum (LDT/PPT, rich in acetylcholine receptors) and the ventromedial medulla (VM) neurons are particularly active. The BF is active in REM and wakefulness and inhibited during NREM.

but not of MT_1KO mice (52, 99), suggesting that MT_1 receptor is involved in circadian regulation.

In keeping, MT_1KO mice show no light/dark differences in circulating corticosterone levels (76), and unlike WT and MT_2KO mice, no light/dark differences in the duration of REM sleep (53). Finally, the abundance of MT_1 compared with MT_2 receptors in the SCN (65) may also suggest a prime implication of MT_1 receptor in circadian regulation.

Further research is necessary to validate this hypothesis linking melatonin, melatonin receptors, circadian rhythms and sleep. Within this context, it will be important to investigate the pathophysiological role of the recently characterized MT1/MT2 heteromers (17), but also of possible heterooligomers between melatonin receptors and 5-HT_{2c} receptors. Notably, 5-HT_{2c} receptors are present in considerable amounts at the level of the SCN (100) and their activation also modulate clock gene expression (101).

CONCLUSIONS AND OPEN QUESTIONS

Melatonin is an important modulator of the sleep/wake cycle by activating MT_1 and MT_2 receptors, even if some authors have also hypothesized that melatonin can have MT_1/MT_2 receptor-independent hypnotic effects (102). Using different experimental approaches, melatonin receptors have been shown to be present in many brain areas/nuclei implicated in the control of the sleep/wake cycle. Importantly, the most recent studies indicate that the two receptor subtypes are differently expressed in regions involved in REM or NREM sleep. For example, the MT_2 is uniquely located in the reticular thalamus, an area involved in NREM triggering. In contrast, the MT_1 receptor is found in the PFH, involved in REM, as well as in the dorsal raphe nucleus and the locus coeruleus, which are either active, slightly active, or silent according to the wakefulness, NREM, and REM sleep stages, respectively. The neural circuits implicated in the regulation of the sleep/wake cycle have yet to be completely elucidated, and may represent an interesting target for the application of the novel technologies of optogenetics and genetic manipulation which would allow for the activation or inactivation of single receptors in specific areas. The current knowledge we have summarized here suggests that the two melatonin receptors subtypes can have either complementary or opposing effects in NREM and REM sleep, likely because of their different expression in brain areas differently implicated in the regulation of the sleep/wake cycle. These findings result mainly result from preclinical studies genetically and/or pharmacologically targeting MT_1 or MT_2 receptors, and partially explain the limited efficacy as hypnotics of melatonin or non-selective MT_1/MT_2 receptor agonists in clinical studies. While the possible role of MT_2 receptor in modulating sleep stages has been confirmed by studies in MT_2 receptor knockout mice and with compounds activating selectively the MT_2 receptor subtype, research on MT_1 receptors is still limited to findings in MT_1 receptor knockout mice. The development of selective ligands for the MT_1 receptor subtype will allow us to test their effects upon the sleep/wake cycle, thus increasing our understanding of the neurobiological role of both MT_1 and MT_2 receptors in sleep.

Most preclinical research investigating the potential hypnotic effects of selective MT_2 agonists/partial agonists has been conducted following only one or a few injections of the drug. No studies have evaluated the effects of a chronic treatment with these different melatonergic compounds on the sleep/wake cycle. This is particularly noteworthy since hypnotics are often prescribed in humans for long periods.

Another important issue arising from the reviewed literature is the importance of considering the time of administration of melatonergic compounds. Comparing preclinical and clinical studies, in humans the treatment has been done early or late (before going to sleep) during the day, and in animals during the light (inactive) or dark (active) phase of the day. Given the circadian variations in the endogenous levels of melatonin and likely in the expression of the two melatonin receptors, it is not surprising that different and/or apparently contrasting findings have been described. Therefore, chronopharmacology should become a *leitmotif* when discussing the potential implications of the novel findings linking the melatonin system to sleep but also to wider biological/pharmacological issues.

The history of pharmacology indeed has taught us that receptor-selective ligands are superior to the respective neurotransmitter itself. For example, serotonin or the precursor tryptophan is less effective than SSRIs for depression or 5-HT2A antagonists for psychosis. Similarly, selective MT_1 or MT_2 ligands may be therapeutically more effective than melatonin in the treatment of sleep disorders.

In conclusions, given the lack of medications specifically registered for treating either NREM or REM sleep disorders and the fact that MT_1 and MT_2 receptors seem to modulate the two sleep stages differently, the future development of selective MT_1 or MT_2 receptor ligands may help to answer this medical need that afflicts a considerable percentage of the population in industrialized countries.

AUTHOR CONTRIBUTIONS

GG and SC conceived the study, collected data, and wrote the review.

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Conflict of Interest Statement: GG is an inventor and assignee of patents for selective melatonin ligands.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Melatonin Synthesis and Function: Evolutionary History in Animals and Plants

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Melatonin is an ancient molecule that can be traced back to the origin of life. Melatonin's initial function was likely that as a free radical scavenger. Melatonin presumably evolved in bacteria; it has been measured in both α-proteobacteria and in photosynthetic cyanobacteria. In early evolution, bacteria were phagocytosed by primitive eukaryotes for their nutrient value. According to the endosymbiotic theory, the ingested bacteria eventually developed a symbiotic association with their host eukaryotes. The ingested α-proteobacteria evolved into mitochondria while cyanobacteria became chloroplasts and both organelles retained their ability to produce melatonin. Since these organelles have persisted to the present day, all species that ever existed or currently exist may have or may continue to synthesize melatonin in their mitochondria (animals and plants) and chloroplasts (plants) where it functions as an antioxidant. Melatonin's other functions, including its multiple receptors, developed later in evolution. In present day animals, via receptor-mediated means, melatonin functions in the regulation of sleep, modulation of circadian rhythms, enhancement of immunity, as a multifunctional oncostatic agent, etc., while retaining its ability to reduce oxidative stress by processes that are, in part, receptor-independent. In plants, melatonin continues to function in reducing oxidative stress as well as in promoting seed germination and growth, improving stress resistance, stimulating the immune system and modulating circadian rhythms; a single melatonin receptor has been identified in land plants where it controls stomatal closure on leaves. The melatonin synthetic pathway varies somewhat between plants and animals. The amino acid, tryptophan, is the necessary precursor of melatonin in all taxa. In animals, tryptophan is initially hydroxylated to 5-hydroxytryptophan which is then decarboxylated with the formation of serotonin. Serotonin is either acetylated to *N*-acetylserotonin or it is methylated to form 5-methoxytryptamine; these products are either methylated or acetylated, respectively, to produce melatonin. In plants, tryptophan is first decarboxylated to tryptamine which is then hydroxylated to form serotonin.

Keywords: melatonin, evolution, antioxidant, biological rhythms, biosynthesis enzymes, endosymbiosis, regulation of melatonin

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INTRODUCTION

After its isolation and identification in the pineal gland of the cow, in subsequent years melatonin was identified in a wide variety of animals and plants (1–7). The extensive distribution of melatonin, especially in the primitive bacteria (cyanobacteria and α-proteobacteria) indicates that the chemical is an ancient molecule that has been retained throughout the evolution of all organisms (8, 9) (**Figure 1**). It is speculated that melatonin evolved in bacteria prior to the process referred to as endosymbiosis. After cyanobacteria and α-proteobacteria were engulfed by early prokaryotes, they eventually evolved into chloroplasts and mitochondria, respectively, such that all unicellular and multicellular organisms ultimately produce this critical indoleamine in these organelles (11–13). With organismal diversification, melatonin universally spread to all organisms and, accordingly, its functions, biosynthetic pathway, generation sites and biosynthetic regulation have also diverged.

The proposed initial function of melatonin was to detoxify free radicals generated during the processes of photosynthesis and metabolism (8, 14–17). With the bio-divergence during organismal evolution, melatonin became a pleiotropic molecule that resists oxidation-related stress but also influences biological rhythms, suppresses inflammation, etc. (18–21).

The genes encoding the biosynthetic enzymes for melatonin have been identified in a number of species; these proteins potentially catalyze different substrates further determining the diverse biosynthetic routes of melatonin (22, 23). The multiple biosynthetic pathways provide direct evidence for melatonin's evolution. From unicellular to multicellular organisms, the subcellular localization of the enzymes related to melatonin biosynthesis may have changed somewhat (24, 25). The separation of the sites of subcellular localization may have been beneficial for the efficient control of melatonin synthesis (22, 26–28).

To exploit the multiple functions of melatonin, organisms developed various mechanisms to regulate its biosynthesis. For example, when faced with stress, activator protein-1 (AP-1), a transcription factor, promotes the synthesis of melatonin via up-regulating melatonin synthesis genes (29–34). Based on its evolutionary history, it seems clear that melatonin not only kept its primary function as an antioxidant but extended its functions to other important biological actions. Moreover, since it co-habitated with other key molecules such as sirtuins for eons, melatonin also learned to functionally cooperate with them (13).

FUNCTIONAL EVOLUTION OF MELATONIN

Molecular oxygen (O_2) began to rise in the Earth's atmosphere (the Great Oxygenation Event) (**Figure 2**) around 2.5 billion years ago due to its persistent release from photosynthetic bacteria that had evolved an estimated billion years earlier (35–37). The rise of atmospheric O_2 was a highly selective pressure for the evolution of organisms to use O_2 as the basis of their metabolism (38, 39). During aerobic metabolism, reactive oxygen species (ROS) are invariably generated when O_2 accepts leaked electrons from the electron transport chain (ETC) (24, 40, 41).

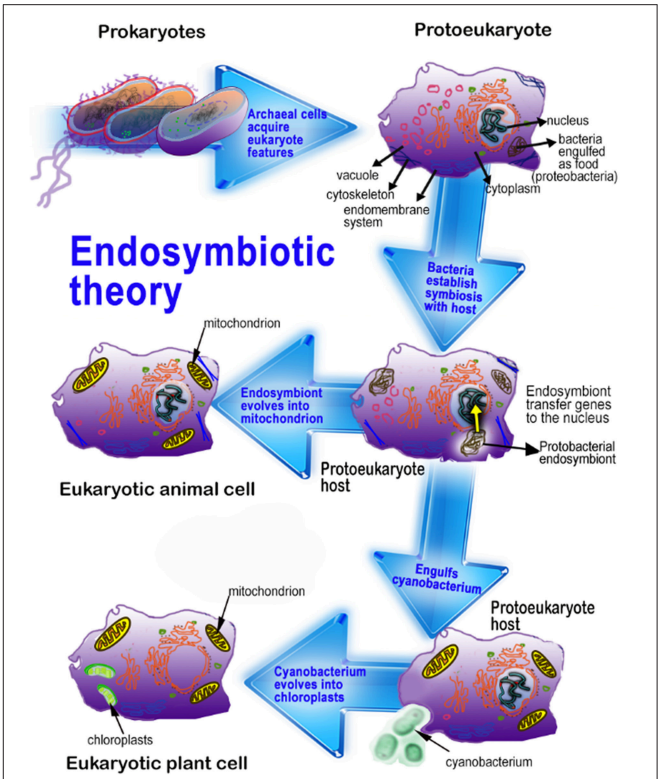

FIGURE 1 | This figure illustrates the endosymbiotic origin of mitochondria and chloroplasts. α-Proteobacteria, originally phagocytized for their nutrient value by early eukaryotes eventually evolved into mitochondria. Photosynthetic cyanobacteria were likewise phagocytized by eukaryotes and eventually formed chloroplasts. Since plants have both mitochondria and chloroplasts, plant cells generally have higher concentrations of melatonin than do animal cells. Adapted from Reiter et al. (10).

It is estimated that up to 4% of the O_2 consumed by organisms during the aerobic metabolism eventually is reduced to ROS (42, 43). These large amounts of ROS are toxic to cells and organisms, inducing the development of complex and effective mechanisms to neutralize them; this initially occurred in early life forms such as bacteria and subsequent unicellular organisms (8, 44). To control oxidative stress, melatonin presumably emerged primarily as an antioxidant and free radical scavenger in early photosynthetic prokaryotic bacteria (12, 13, 45, 46). Melatonin has retained, until the present time and in all organisms, its ability to control oxidative stress that results from free radical production that occurs during photosynthesis and respiration (8, 47–49).

The special structure of melatonin determines its high efficiency in detoxifying free radicals based on its ability to donate an electron or a hydrogen atom, or depending on the radical type, potentially by other means as well (5, 15, 17). The superior antioxidant capacity of melatonin to limit oxidative stress is, at least partially, attributed to what is referred to as the cascade reaction which occurs when it generates derivatives that are likewise free radical scavengers (12, 15, 50–52). Melatonin interacts with a variety of ROS to produce cyclic 3-hydroxymelatonin and other melatonin metabolites,

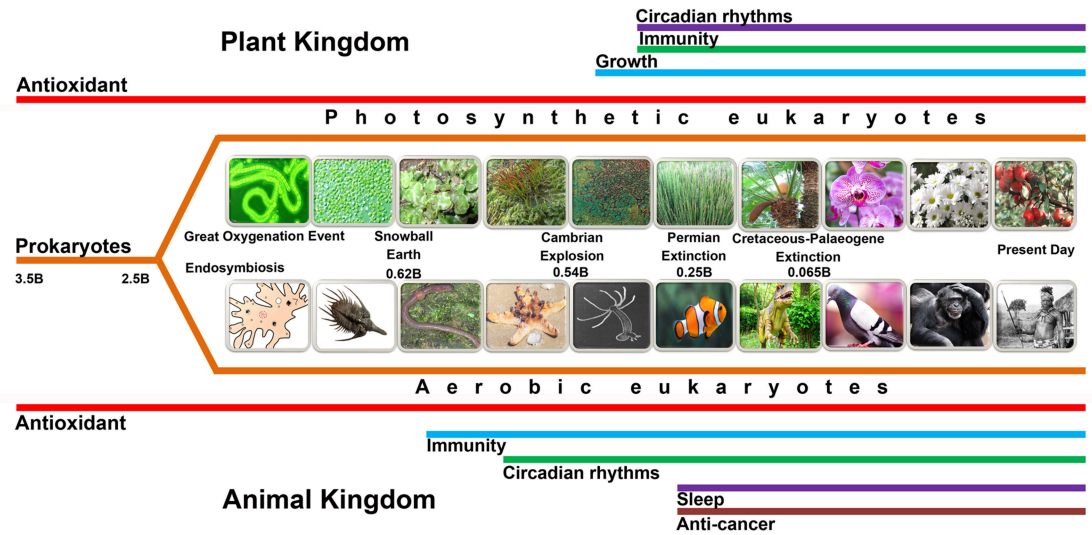

FIGURE 2 | This figure summarizes the possible evolution of various functions (not all are depicted in this figure) of melatonin. Melatonin, predictably, initially evolved in bacteria for the purpose of mitigating oxidative stress, i.e., as an antioxidant (red lines). When the bacteria were phagocytized as food by early eukaryotes, they eventually developed a mutually beneficial association with their hosts and evolved into mitochondria and chloroplasts (see **Figure 1**); this series of events is referred to as endosymbiosis. Subsequently, as evolution proceeded, mitochondria (animals and plants) and chloroplasts (plants) were preserved up until the present day. Thus, mitochondria and chloroplasts of every species that has ever existed or exists today, we theorize, presumably produce melatonin. This presumption is supported by recent findings which show that these organelles, in many cases, possess the necessary synthetic machinery to generate melatonin. Melatonin's role as an antioxidant in these organelles is of great importance since they are sites of major free radical production. Other colored lines, which are appropriately labeled, identify other functions of melatonin. It is essential that the time frame for these functions, as illustrated by the length of the colored lines, do not accurately depict the time of evolution of these functions. Major events in the history of the Earth are also identified. The "B" following the numbers refers to "billions of years ago.".

e.g., *N*1-acetyl-*N*2-formyl-5-methoxykynuramine and *N*-acetyl-5-methoxykynuramine (53–56). These metabolites function as radical scavengers, sometimes even more aggressively than melatonin regarding their capacity to neutralize ROS (15, 57).

Despite its very long evolutionary history and its multiple functions, the chemical structure of melatonin has remained unchanged for billions of years (13). Moreover, melatonin may have been retained by all organisms even with their very wide biodiversification during evolution. This relates to the conservation of mitochondria and chloroplasts (or both) in most cells of all organisms. One exception is red blood cells which, during erythropoiesis, eject certain organelles including mitochondria.

As already noted, melatonin originally exclusively functioned as an antioxidant in primitive bacteria; however, over billions of years of evolution it became a pleiotropic molecule in multicellular organisms (**Figure 2**). The development of new functions of melatonin logically expanded the spectrum of its antioxidant activity (8). Regulation of biological rhythms is one of the key functional extensions. In early primitive unicellular plants and animals, more free radicals were produced during the photophase than during the scotophase; thus, larger amounts of melatonin were presumably consumed during the detoxification of excessively-produced free radicals during the day (58, 59) resulting in a diurnal rhythm of melatonin. In contrast to unicellular organisms, which directly perceive photoperiodic changes and synchronize their biological activities accordingly (60), complex multicellular organisms could no longer respond directly to the photoperiodic changes (24). A signaling molecule, therefore, was required to ensure the photic information was transduced into a circadian signal for all cells (61). The alteration in melatonin levels in bacteria due to its differential utilization as a scavenger accurately reflected the photoperiodic changes of the light/dark cycle; theoretically, multiple organisms adopted the melatonin cycle as a signaling system for this purpose (24, 62, 63).

Multicellular organisms, therefore, co-opted a melatonin rhythm that already existed; but rather than depending on the metabolic utilization of melatonin to determine the cycle, they developed the subcellular framework to produce more melatonin during the scotophase than during the photophase, thereby ensuring a day:night melatonin cycle. In most vertebrates, but seemingly not all (64), this required the evolution of the pineal gland which is the location of the circadian production and, importantly, cyclic secretion of melatonin allowing all cells access to light:dark information. In present day animal species, the melatonin cycle, with highest levels at night, is the same regardless of the activity pattern of the species, i.e., nocturnal, diurnal or crepuscular. In addition to the neural connections between the eyes and the pineal gland, most clearly described in mammals, the pineal of some lower vertebrates responds directly to light stimuli (65). While the photic information has a direct impact on the electrophysiology of the organ, there is no proof that it alters melatonin production or secretion. In non-vertebrate animals and in plants, much less is known about the circadian production of melatonin (66, 67), although

these species do exhibit other circadian rhythms (68, 69) as well as possible 24 h fluctuations in melatonin, but sometimes the highest levels occur during the day (70).

Other actions of melatonin that have evolved and relate to the antioxidant activity of melatonin include retarding some age-related processes, anti-inflammatory activity, resisting neurodegenerative changes, the prevention of apoptosis in normal cells, and the preservation of mitochondrial and chloroplast physiology (8, 71–74) (**Figure 2**). These functions are associated, at least in part, with melatonin's ability to neutralize free radicals.

The actions of melatonin in different species have clearly diverged during the differentiation of major animal and plant taxa. These functions show a close relationship with the characteristics of the specific taxa. In mammals, melatonin is a molecule with hormonal properties (10, 75–77). The hormonal properties of melatonin are apparent in the regulation of seasonal reproductive activity, facilitation of sleep physiology, promotion of immunoresponsiveness, suppression of carcinogenesis, promotion of stem cell proliferation, anti-inflammation, and modulating aging (73, 78–83). Some of these actions are surely mandated by the interaction of melatonin with cell membrane receptors, i.e., MT1 and MT2, and/or perhaps with nuclear binding sites and are, therefore, considered hormonal (see below). For example, melatonin's ability to constrain cancer cell proliferation often involves membrane receptors (84, 85). There is also evidence, however, that receptor-independent actions such as its free radical generating capacity ("pro-oxidant"), an action possibly unique to cancer cells (86), also kills tumor cells (87, 88). Moreover, melatonin's multiple means by which it limits cancer metastases have not been unambiguously shown to be receptor-mediated (89). Thus, in mammals, melatonin is not a typical hormone and functions via receptor-dependent and receptor-independent means. On the evolutionary scale, the free radical scavenging properties, which continue to exist in mammalian cells, preceded the evolution of receptors for this indoleamine; thus, the initial actions of melatonin were receptor- independent.

In early non-mammalian vertebrates, the pineal organ directly responded to light that penetrated a cartilaginous plate overlying the epithalamus (90). This photic information was detected by photoreceptive elements similar to those in the retinas, with the electrical messages being sent to adjacent neural structures (91). In higher vertebrates, the pineal gland is no longer directly light-sensitive, although it does contain evolutionary morphological remnants of photoreceptive rods/cones (92), but it remains influenced by light and darkness via complex retina-suprachiasmatic nucleus-sympathetic neural connections (93).

The mammalian pineal gland probably did not evolve as nor should it be strictly classified as an endocrine gland. Endocrine glands are typically regulated by the secretory products from other glands and exhibit either feedback or feedforward responses when contacted by these agents. Also, because of their primary regulation by hormones, hormone production and secretion typically are only modestly impacted by depriving endocrine glands of their sympathetic innervation. These features are in marked contrast with those of the pineal gland, where other hormones have barely perceptible effects on pineal melatonin production (94, 95). The sympathetic denervation destroys the function of the pineal (96), while for other endocrine organs (e.g., anterior pituitary, thyroid gland) denervation is essentially inconsequential. Giving norepinephrine or isoproterenol to animals in which the pineal has been sympathetically denervated induces a rapid increase in pineal melatonin synthesis indicating that pineal synthetic processes are under the control of the nervous system rather than by hormones (97); there may, however, be some unique experimental conditions that perturb melatonin synthesis in the pineal gland independent of its innervation (98); how these actions are mediated remain unknown. For example, high melatonin production can be shifted to immune competent cells (99) under inflammatory conditions. This important observation was unanticipated and opens up the possibility that there may be other conditions under which melatonin synthesis is enhanced in other cell types.

Another argument against melatonin being exclusively a hormone comes from the observation that an estimated 99% of the melatonin in vertebrates is likely not produced in the pineal gland and is never released into the circulation. The discovery of melatonin in mitochondria, where it likely functions as a direct free radical scavenger and as an indirect antioxidant, means that the total quantity of melatonin synthesized in vertebrates is much greater than originally envisioned. In addition to functioning as a scavenger at the site at which it is produced, i.e., mitochondria, melatonin generated at the subcellular level may be locally released to function as a paracrine or autocrine agent (100). There is also preliminary evidence that mitochondria-produced melatonin is discharged from this organelle after which it interacts with receptors on the outer mitochondrial membrane where it may influence the release of cytochrome c (101) (**Figure 3**). In invertebrates (104) which lack a pineal gland, endogenous melatonin production may respond to external environmental alterations that do not involve the light:dark cycle.

In the plant kingdom, melatonin is demonstrated to be a multi-regulatory molecule with diverse functions in plant growth and development, such as seed protection and germination, root development, fruit ripening, and senescence (105–108). Compared to animals, plants face more environmental challenges because of their sessile nature. As a protection against these stresses, they rapidly unregulate melatonin synthesis which then functions in the protection against oxidative stress induced by these challenges (109). As mentioned, melatonin works independent of receptors when it clears ROS (110, 111). The major membrane melatonin receptors in animals, MT1 and MT2, activate different signaling cascades to improve or antagonize biological effects (112–115). To date, only one phytomelatonin receptor (CAND2/PMTR1) has been identified; it regulates stomatal closure via a H_2O_2 and Ca^{2+}signaling transduction cascade (116). In addition, phytomelatonin can interact with unknown receptors with active H_2O_2/NO signaling pathways, and further improve plant stress tolerance by activating a variety of antioxidant enzymes, alleviating photosynthesis inhibition, and modulating transcription factors; these transcription factors are involved with stress resistance, chelating and promoting transport of heavy metals, or activating other stress-relevant

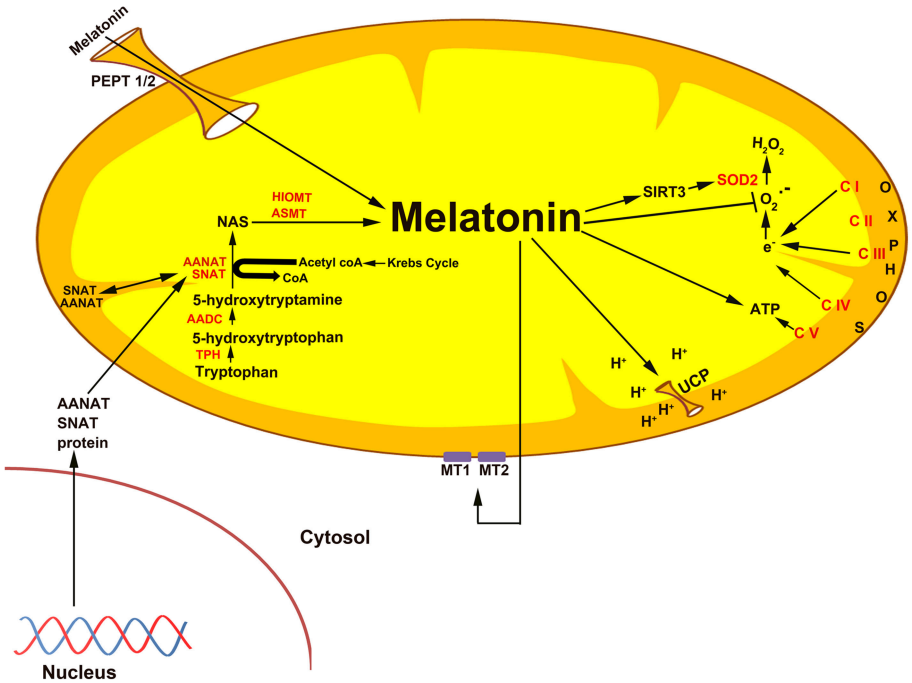

FIGURE 3 | The association of melatonin with mitochondria is predicted on the basis of the origin of these organelles as specified in the text. Current evidence suggests that melatonin is synthesized in some species in the mitochondrial matrix as illustrated here. Also, exogenously administered melatonin concentrates in the mitochondria (102), i.e., melatonin is a mitochondria-targeted agent. Given that melatonin functions as an antioxidant is particularly important in mitochondria since these organelles are a major site of free radical generation. In addition to directly neutralizing reactive oxygen species, melatonin also stimulates the antioxidant enzyme superoxide dismutase (SOD2), an action that involves an elevated level of sirtuin 3 (SIRT3) (39). Melatonin potentially enters mitochondria through the oligopeptide transporters, PEPT1/2 (103). Melatonin also influences mitochondrial membrane potential by influencing uncoupling protein (UCP). Also, melatonin from the matrix may leak out of the mitochondria to interact with the melatonin receptors, MT1 and MT2, to control the release of cytochrome c.

hormones such as salicylic acid, ethylene, and jasmonic acid (109, 117, 118). In animals, melatonin also binds directly to the catalytic site of quinone reductase 2 (QR2, E.C. 1.10.99.2), a cytosolic molecule, to modulate the activity of this enzyme; that modulation may be either up or down regulation (119, 120). Importantly, the change in QR2 activity may further play a key role in ROS generation or detoxification (121).

ORIGIN OF MELATONIN RECEPTORS

The presumed original function of melatonin, i.e., as a direct free radical scavenger, required nothing of the cell except positioning melatonin in close proximity to where the bulk of the ROS are usually formed. Such positioning of an antioxidant is essential since free radicals have an extremely short half-life and instantaneously damage molecules in the immediate neighborhood of where they are formed. If a free radical scavenger is not properly situated, it cannot prevent the initial damage inflicted by a highly reactive radical. To accomplish this proper placement, evolution arranged for the uptake by and synthesis of melatonin in mitochondria (101, 102) and chloroplasts (108, 122), both major contributors to the total oxidative burden of cells.

In currently-surviving vertebrates, melatonin has a very extensive physiological toolkit. To broaden its functional repertoire, it was necessary for its binding sites/receptors and associated signaling transduction processes to also evolve. Many of the currently known activities of melatonin are mediated by G-protein coupled receptors in the membranes of animal cells (113, 123, 124). The best known receptors associated with cell membranes are members of the G-protein coupled receptor family (111, 125, 126); they are designated MT1 and MT2 (127–129).

Perhaps, of special relevance to the current discussion, is the finding that the MT1 receptor, generally considered to be confined to the limiting membrane of cells has also been recently associated with the outer membrane of mitochondria (101). According to the researchers who made this discovery, melatonin from the mitochondrial matrix diffuses out of these structures and interacts with MT1 receptors on the outer membrane of these organelles; they coined the term "automitocrine" to define this process. Via this receptor-mediated pathway, mitochondria-generated melatonin may control the release of cytochrome c from the matrix. This self-regulatory process has implications for apoptosis resulting from extensive free radical damage.

In addition to the well-characterized and highly relevant cell membrane receptors which are indispensable for a number of

melatonin's key functions, there are also binding sites in the cytosol (130) and in the nucleus (131–133). In the cytosol, the enzyme quinone reductase 2 (QR2) has been designated as receptor MT3 (134). The activity of this detoxifying enzyme may to be related to some of the actions of melatonin in reducing oxidative damage. Melatonin also couples with calmodulin in the cytosol, an action that is reportedly linked to the cancer-inhibitory effect of the indoleamine (135, 136).

In an invertebrate, the crayfish (*Procambarus clarkii*), melatonin functions in the modulation of the reticular photoreceptor potential amplitude with the intensity change differing between the day and night (137). With the aid of the commonly used melatonin receptor blockers, the authors deduced that the actions of melatonin on visual photoreceptors are mediated by a site reminiscent of the mammalian MT2 receptor. In another crustacean, the crab (*Neohelice granulata*), some of the metabolic actions of melatonin are inhibited by luzindole, a classic MT1/MT2 receptor blocker (138).

In the honey bee, *Apis cerana*, a melatonin receptor with the typical seven transmembrane domains has been characterized (139); the authors named it AccMTNR1A. It mediates the response of this species to cold stress, a feature that is common with that of plants. Silencing of the receptor also interfered with the transcription of some antioxidant signaling pathways. This illustrates that antioxidant enzyme activity may be regulated in the honey bee as they are in plants (105). Collectively, the data from invertebrates show that not only do they produce melatonin but they probably have receptors that mediate some of its actions. Fossil records indicate that insects evolved more than 400 million years ago, so the melatonin receptor has likely existed for at least the same time duration.

Tetrahymena are nuclear dimorphic, unicellular, ciliated eukaryotes. Like many other eukaryotes, *Tetrahymena* feed on bacteria so this evolutionarily-early organism would be expected, due to endosymbiosis, to have retained the melatonin synthetic potential of the engulfed bacteria. There is evidence that *Tetrahymena* contain biogenic amines including possibly melatonin (140). Whether this species possesses melatonin binding sites/receptors has not been established. If they are found to contain melatonin-binding molecules, it would show that some type of melatonin receptor evolved about one million years after melatonin arose. As it currently stands, there is little information related to when melatonin receptors originated.

As noted above, melatonin was discovered in land plants about 25 years ago (2, 3) where it functions as an antioxidant in a receptor-independent manner (141) as in animals. The first phytomelatonin receptor, designated CAND2/PMTR1, was described in *Arabidopsis thaliana* (116). This receptor is located on epithelial cells which govern the closure of the leaf stomata. For this process, the signal transduction cascade involves Gα subunit–activated H_2O_2 production and Ca^{2+} signaling. It is estimated that land plants came into existence on Earth about 200 million years ago. Whether the first land plant that appeared or any plants that preceded them possessed melatonin receptors is unknown.

In addition to the direct scavenging of radicals and radical products by melatonin, land plants also have many of the antioxidant enzymes that exist in animals. In plants, the enzymes are melatonin-influenced and are quickly upregulated when the plant is exposed to an abiotic stress, e.g., draft, heat, cold, toxin, etc. (105, 142). It is presumed that this upregulation involves melatonin receptors as is likely the case for animals as well.

For additional details on the pharmacological characterization, cloning and signal transduction pathways of melatonin receptors, the reader is referred to comprehensive reviews of this subject by the groups of Jockers et al. (114, 115), Oishi et al. (130), Tosini et al. (143), Liu et al. (128), Dubocovich (114). Obviously, melatonin receptors developed subsequent to the evolution of melatonin. Based on what is currently known of their distribution, they probably originated with the origin of multicellular organisms, both plant and animal.

DIVERGENCE OF MELATONIN BIOSYNTHESIS IN DIFFERENT TAXA

Melatonin is believed to exist in all living organisms including bacteria, yeasts, fungi, animals, and plants (144, 145). This molecule is formed exclusively from the amino acid tryptophan (146). While tryptophan is consumed in the diet, it can also be synthesized via the shikimic acid pathway starting with D-erythrose-4-phosphate, phosphoenolpyruvate, or carbon dioxide in some species (147). With the evolution of organisms (apart from animals) bacteria, fungi, and plants retained the ability to synthesize tryptophan (24). Conversely, mammals only attain tryptophan, an essential amino acid, during food intake. A reduction of tryptophan leads to the marked lowering of melatonin production in animals compared to that in plants (145, 148). Since plants cannot behaviorally avoid extremely stressful conditions, they require extra protection from stress; hence, the biosynthesis of tryptophan is presumably retained in plants to ensure that melatonin is available for relieving oxidative stress levels under environmentally-stressful conditions.

Beginning with tryptophan, melatonin biosynthesis includes four enzymatic steps in all organisms (22, 67). During its evolution lasting billions of years, the pattern of the melatonin synthesis became diversified (**Figure 4**). Tryptophan is first converted to serotonin which involves decarboxylation and hydroxylation. There are two strategies for the synthesis of serotonin that leads to melatonin production in different taxa. The biosynthetic pathway of serotonin in microorganisms and plants is different from that of vertebrates. Tryptophan is decarboxylated to tryptamine by tryptophan decarboxylase (TDC), followed by serotonin biosynthesis catalyzed by tryptamine 5-hydroxylase (T5H) in plants (149, 150). In contrast, rather than tryptophan decarboxylation being the initial step in serotonin production, animals first hydroxylate tryptophan using tryptophan hydroxylase (TPH) to form 5-hydroxytryptophan and then 5-hydroxytryptophan is decarboxylated by aromatic amino acid decarboxylase (AADC) to form serotonin (7).

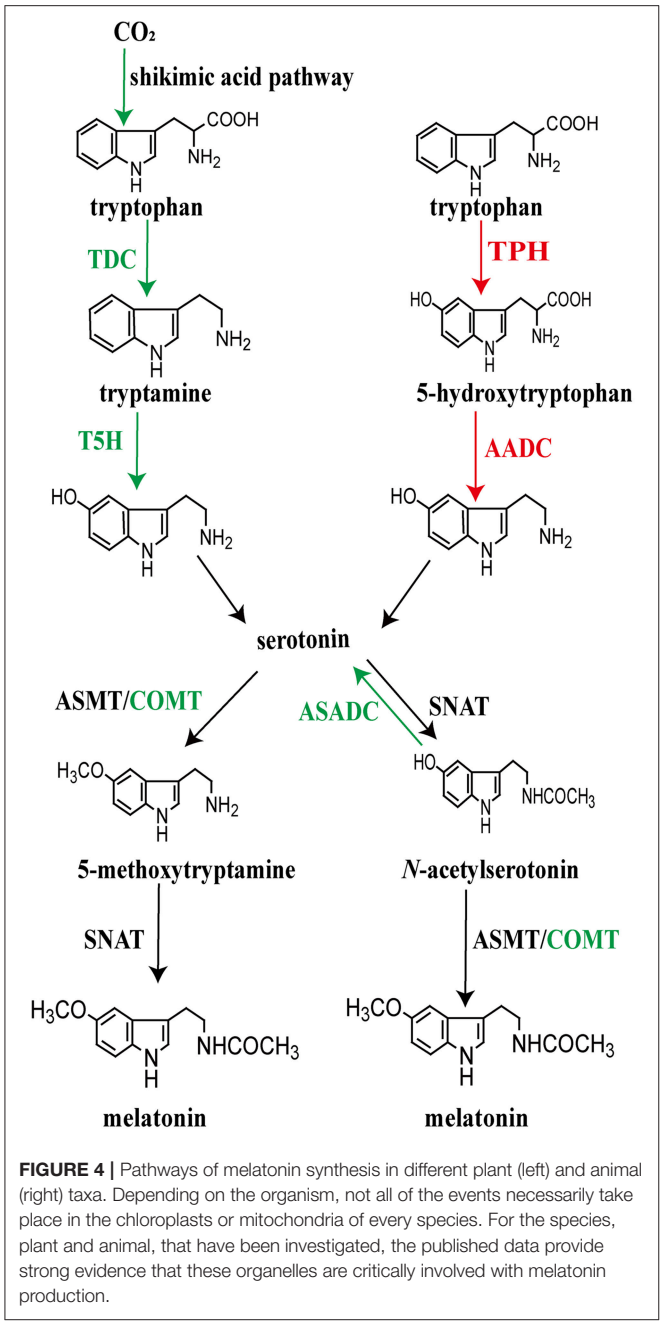

FIGURE 4 | Pathways of melatonin synthesis in different plant (left) and animal (right) taxa. Depending on the organism, not all of the events necessarily take place in the chloroplasts or mitochondria of every species. For the species, plant and animal, that have been investigated, the published data provide strong evidence that these organelles are critically involved with melatonin production.

Between tryptophan and melatonin, serotonin is a key intermediate after which the biosynthetic process utilizes two potential pathways, each of which includes two consecutive enzymatic steps to generate melatonin (15, 22). These steps catalyze serotonin to form the final product, melatonin; this involves serotonin *N*-acetyltransferase (NAT) and *N*-acetylserotonin *O*-methyltransferase (ASMT; formerly known as hydroxyindole-*O*-methyltransferase, HIOMT) (151–155). The penultima enzyme, NAT, plays a key role in the conversion of serotonin to *N*-acetylserotonin while the last enzyme, ASMT, catalyzes NAS to produce melatonin (153, 154, 156). Alternatively, serotonin can be first O-methylated to 5-methoxytryptamine (5-MT) by AMST; thereafter, 5-MT is N-acetylated by NAT to produce melatonin (7, 155). Differing from the formation of serotonin, the two alternative pathways for the conversion of serotonin to melatonin, likely occur in both plants and animals as well as in the microorganisms. However, different homologs of NAT have been detected between plants and animals, as well as ASMT in the two groups, revealing their different origins during evolution (122, 157–159). NAT seems to have originated independently as indicated by the few shared amino acid residues between animals and plants as well as between primitive cyanobacteria and archaea (24, 158). Thus, the enzymes controlling the biosynthetic steps of melatonin seem to have different origins at the emergence of melatonin; these enzymes further evolved divergently after endosymbiosis.

As already mentioned, the intermediate processes in melatonin production display a variety of differences among taxa. The present-day organisms possess diverse melatonin biosynthetic pathways. The enzymes that produce melatonin may play roles in catalyzing different substrates. Beyond the four key enzymes, other evolved enzymes are reported to directly participate in the biosynthesis of melatonin. This is supported by evidence that plants evolved caffeic acid O-methyltransferase (COMT), which is involved in the synthesis of 5-methoxytryptamine/melatonin by methylating serotonin/*N*-acetylserotonin (160). Apart from the classic enzymes strictly required for the melatonin biosynthetic pathways, Lee et al. (161) found that *N*-acetylserotonin can be converted to serotonin in rice seedlings by *N*-acetylserotonin deacetylase (ASDAC), which may result in a reduction in the content of melatonin (**Figure 4**).

SUBCELLULAR LOCALIZATIONS OF ENZYMES ASSOCIATED WITH MELATONIN BIOSYNTHESIS FROM THE VIEW OF ENDOSYMBIOSIS

Based on the endosymbiotic theory, when the early eukaryotic cells (having nuclei but no mitochondria) endocytosed α-proteobacteria or cyanobacteria (**Figure 1**), rather than digesting these bacteria, the proto-eukaryotic cells developed a symbiotic association with them (162–164). The observations that the NAT protein, the rate-limiting enzyme of melatonin synthesis, is abundantly located in mitochondria of animals and chloroplasts of plants further support the different origins of the melatonin biosynthetic enzymes as a result of endosymbiosis (26, 165, 166). Furthermore, the DNA sequences and protein residues of cyanobacterium, a plant-type species, and rice are closely related, implying that the plant NAT gene was likely endosymbiotically-derived from cyanobacteria (167, 168). NAT genes of other eukaryotic organisms including fungi, invertebrates, and vertebrates seemingly evolved from *Rhodospirillum rubrum* (the presumed precursor of mitochondria) or closely related species since their NAT genes share similarity to some extent (24, 122, 169).

With endosymbiotic evolution, the function of melatonin synthesis was carried into multicellular organisms. Thus,

mitochondria and chloroplast, which resulted from the endosymbiosis of α-proteobacteria or cyanobacteria, respectively, became the major melatonin generating subcellular organelle in both animals and plants (165, 166). In terms of function, melatonin produced in these two organelles most likely detoxifies excessive ROS and reactive nitrogen species (RNS) generated during oxidative phosphorylation and other metabolic actions (55, 73). The production of melatonin in mitochondria provides maximal on-site protection of these critical organelles (**Figure 4**). In plants, melatonin in chloroplasts provides a similar defense against oxidative stress with subsequent evolution after endosymbiosis. The melatonin-associated genes of the incorporated bacteria were gradually transferred from both mitochondria and chloroplasts to the nuclear genome of each host (24, 164–166, 170). While mitochondria and chloroplasts are considered major sites of melatonin synthesis, it does not preclude the possibility that some melatonin is not also formed in the cytosol ((171); also, see below).

With subsequent evolutionary processes, melatonin-related genes were modified by mutations and in response to natural selective pressures in different species (24, 155, 172). Specifically, the major structural differences of the melatonin synthases among phylogenetically distant species are the regulatory regions which could further influence the subcellular localization of these proteins (7, 24). At least in plants, the subcellular localization analysis documented that the rate-limiting enzyme for melatonin synthesis, NAT, is found in both chloroplasts and mitochondria (26). TPH, ASMT/COMT, and TDC/AADC locate in the cytoplasm while T5H is distributed in the endoplasmic reticulum (7, 22, 150, 154). This subcellular location of melatonin synthesis enzymes suggests that during evolution (**Figure 2**), the sites of melatonin synthesis became more diverse and extended to the cytoplasm and endoplasmic reticulum (22). This divergent distribution of melatonin production shows a good relationship with the transformation from prokaryotic cell to eukaryotic cell. Regarding the efficiency of melatonin biosynthesis, the present biosynthetic model is consistent with adequate substrate availability. For example, acetyl-CoA, a key substrate for melatonin production, is synthesized in the mitochondria through pyruvate dehydrogenase complex reaction (39, 173, 174). Furthermore, the different subcellular sites of melatonin synthetase avoid substrate competition by other enzymes preferring the same substrate (13, 22, 175). Thus, multiple subcellular sites of melatonin biosynthesis in both plants and animals could promote synthetic efficiency of this essential molecule (171).

MULTIPLE MECHANISMS PRECISELY REGULATE MELATONIN BIOSYNTHESIS

The presence of melatonin at several sites correlates with its biological functions. As in animals, 24-h rhythms have been described in plants, e.g., in *Chenopodium*, which shows a nocturnal maximum growth rate around light/dark transition (176). Also, the dinoflagellate *Lingulodinium* and numerous other microalgae including chlorophyceans (like plants, members of viridiplantae), exhibit robust circadian rhythms of melatonin (177). In comparison to animal cells, plant cells contain much higher levels of melatonin, probably because they have two melatonin producing organelles, mitochondria and chloroplasts (77, 158, 178). In some species such as in *Glycyrrhiza uralensis*, cranberry and several medicinal herbs melatonin levels are reportedly several orders of magnitude higher than those in the serum of animals. Remarkably, the levels of melatonin in the pistachio nut may reach 230 μg/g (15, 145, 179, 180). This may relate to the high environmental stress condition under which this plant normally grows. The immobility of plants results in them being subjected to more unavoidable environmental stressors, causing elevated ROS production and oxidative damage. Thus, they require additional protection from stressors by means of intrinsic mechanisms including high levels of endogenously-produced antioxidants, such as melatonin (158). This speculation is supported by the observation that a variety of environmental insults induce a dramatic increase in melatonin levels in plants (24, 141, 181). Under some conditions, stressful situations may also induce melatonin production in animals, e.g., physiological ischemia/reperfusion events (182). Also, plant cells generally have higher levels of melatonin than animal cells; this likely relates to the fact that plant cells have two sources of melatonin (mitochondria and chloroplasts) while animal cells have a single source.

The presence of melatonin in both plants and animals raises the question as to whether animals and plants have different mechanisms for modulating the biosynthesis of this indole-containing compound. Current data indicate that the regulatory mechanisms of melatonin synthesis are fundamentally different between animals and plants (**Figure 5**). In vertebrates, melatonin is referred to as the chemical expression of darkness (based only on pineal and blood levels); in general, in plants a day/night rhythm is less common, although not totally absent in some species, with the melatonin concentrations sometimes not varying much throughout the light:dark cycle (24, 183, 184). Hence, melatonin synthesis in some plants is non-rhythmic, as in the mitochondria of animals (101).

In vertebrates, light detection by the retinas suppresses the activity of NAT and melatonin production (185, 186). In plants, there is positive correlation between light intensity and melatonin levels with plants growing in habitats exposed to high light intensities, such as Mediterranean or alpine environments, usually having higher melatonin levels than the same or related species growing in other locations (187). In some species, melatonin was reported to be enhanced by darkness of even short duration, e.g., 1 h in rice seedlings (188), findings supported by an elevated ASMT expression in response to darkness (189). Moreover, heat-induced elevation of melatonin was antagonized by light in Oryza (188). In addition to these exceptions, the signal transduction pathways and regulatory mechanisms in vertebrates differ substantially from those in plants. The major regulatory components of melatonin synthesis in mammals, e.g., norepinephrine and its receptors, are not detected in plants and are unlikely to exist, indicating the loss of this pathway in the plants during evolution (24).

As noted above, the biosynthesis of melatonin is closely associated with four successive enzymes leading to the production of this compound. NAT usually is believed to

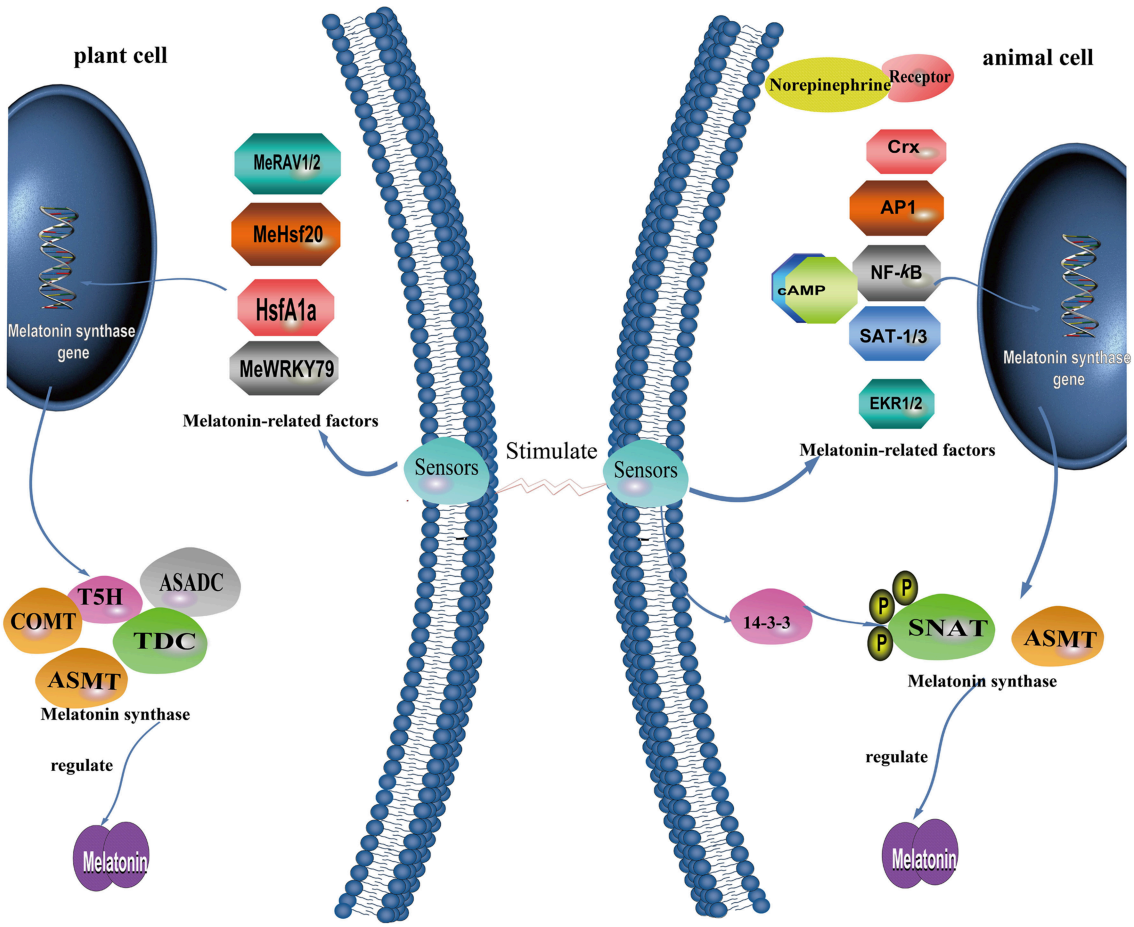

FIGURE 5 | A summary of what is known concerning the molecular mechanism governing melatonin production in plant cells and animal cells.

be the rate-limiting enzyme in pineal melatonin synthesis in vertebrates, although ASMT may limit this level around the nocturnal melatonin maximum (190). For plants, under most circumstances, NAT activity correlates well with the quantity of melatonin produced. In rice seedlings, however, melatonin was reported to be highest at the time of enhanced ASMT expression (191, 192). Typical for animals, the activities of NAT and melatonin production usually exhibit a good positive relationship (185, 193). The regulation of NAT in the pineal gland depends on the animal taxa examined. In many mammals, NAT gene expression in the pineal is either up- or down-regulated by the signals received from the suprachiasmatic nucleus (SCN) while in some species with a predominantly transcriptional control of NAT, its mRNA levels are stimulated up to two orders of magnitude (194). For primates and ungulates, NAT is mainly controlled post-translationally by phosphorylation and dephosphorylation, and stabilization of the phosphorylated form by a 14-3-3 protein (195). While the four successive enzymes positively regulate the pineal concentration of melatonin in all organisms, the overexpression of melatonin biosynthetic genes, such as *TDC*, *NAT*, and *ASMT* do not always lead to the accumulation of melatonin in plants (33, 122, 155, 159, 196, 197). Recently, the *ASDAC* gene was found to catalyze the conversion of *N*-acetylserotonin to serotonin, a reverse reaction from the usual melatonin biosynthetic pathway in plants (161). Clearly, plants possess two genes encoding both NAT and ASDAC proteins, which impact the biosynthesis of melatonin; NAT favors melatonin synthesis, whereas ASDAC lowers melatonin levels.

Especially in plants, melatonin is maintained at a relatively constant level under normal conditions; however, it can be greatly and rapidly upregulated in response to unfavorable conditions such as cold, heat, salt, drought, oxidative and nutrient stress, and bacterial infection (142, 198–203). The underlying mechanisms for the rapid regulation of melatonin production have not been identified including the translation and post-translational regulation of melatonin synthesis enzymes, and the upstream transcription factors of these rate-limiting enzymes or isoenzymes (204, 205). Melatonin biosynthesis genes may have a role at the transcriptional level to control the content of melatonin (**Figure 5**). For this process, different taxa evolved divergently to work with other factors for self-development or coping with stressful conditions.

Activator protein-1 (AP-1) is a stress-responsive transcription factor that can be regulated by oxidative stress in many cell types (206). AP-1 seems to promote both NAT and ASMT activities to enhance melatonin synthesis. Structural analysis of the human *ASMT* gene promoter shows that it contains an AP-1 site at position−166 and similarly, there is an AP-1 transcription factor-binding site in the *AANAT* mouse gene (29, 30). Interestingly, stress uniformly stimulates glucocorticoid production in organisms (207). Glucocorticoids upregulate the transcriptional activity AP-1 and thereby promote gene expression for melatonin synthesis (171, 208). AP-1, in addition to acting as a signal transducer and activator of transcription-1 and 3 (STAT-1; STAT-3), competes with NF-κB for binding to nat-κB1 to regulate the transcription of NAT (32). Transcription of NAT driven by NF-κB dimers mediates pathogen-associated molecular patterns (PAMPs) or pro-inflammatory cytokine-induced melatonin synthesis in macrophages by binding to one or two upstream κB binding sites (nat-κB1 and nat-κB2) of the NAT promoter in RAW 264.7 cells (209). LIM homeobox transcription factor Isl1 positively modulates melatonin synthesis by targeting NAT at the (ATTA/TAAT) motif, via the ERK signaling pathway of norepinephrine (34). Regarding the binding site of TFs, the chicken NAT gene does not contain a canonical cAMP-response element (CRE) sequence TGACGTCA (210) but a TTATT8 repeat sequence and a CLS (6/8 identical to the canonical CRE) in basal and cAMP-driven promoters which bind c-Fos, JunD, and CREB to enhance basal and forskolin-stimulated NAT transcription (211). This motif was not found in NAT genes from other species, including mouse, rat, and zebrafish (211). For the rat, a cAMP- response element (CRE) -like sequence (CLS; TGCGCCA)-CCAAT complex in the flanking region and a canonical CRE in the first intron drives cAMP-dependent induction of the NAT gene as well (212, 213). In addition, the cone-rod homeobox (Crx) transcription factor was reported to regulate the expression of NAT in the mouse pineal gland.

For plants, limited information related to transcriptional regulation is available compared with that of animals. Evidence from recent studies show that a multifunctional enzyme, namely caffeic acid O-methyltransferase (COMT), can also catalyze the last step of melatonin biosynthesis (28). In rice, melatonin biosynthesis requires ASMT or COMT activity (154). Cai et al. (33) revealed that, in tomato, cadmium stress induces the expression of HsfA1a, which acts as a positive regulator of COMT1 transcript levels by binding to the COMT1 gene promoter heat-shock element (HSE) sequence (GAANNTTC), and induces melatonin accumulation. For cassava, three TFs were found to modulate melatonin biosynthesis. Cassava bacterial blight induces the expression of MeWRKY79 and MeHsf20, which activate the expression of MeASMT2 via binding to W-box (TTGACC/T) and HSEs (GAAnnTTC) in the MeASMT2 promoter; this, in turn, increases melatonin accumulation and confers improved disease resistance (205). MeRAV1 and MeRAV2 may directly regulate three melatonin biosynthesis genes (*MeTDC2*, *MeT5H*, and *MeASMT*) by binding their promoter containing CAACA motif as transcriptional activators, and thus up-regulate melatonin biosynthesis in response to disease resistance against cassava bacterial blight (214).

CONCLUSION REMARKS

The acquisition of additional functions by melatonin, which is believed to have originally evolved to provide molecular protection from free radicals, occurred over a very long evolutionary period. It is theorized that melatonin first appeared in bacteria about 3.0–2.5 billion years ago. When these melatonin-synthesizing bacteria were phagocytized by early eukaryotes as food, over time they established a symbiotic association with their hosts and developed into mitochondria and chloroplasts. Since the bacteria that were ingested had the ability to synthesize melatonin, this important function was retained by the mitochondria and chloroplasts. As a consequence, we hypothesize that these organelles have produced melatonin in every plant and animal species that has ever existed and that this occurs in present day animal and plant cells as well. Thus, every cell that possesses mitochondria (animals and plants) or chloroplasts (plants), we feel, has the capacity to produce melatonin. Melatonin at these sites is important to provide protection against free radicals which are abundantly generated in these organelles. Over its very long evolutionary history, melatonin has acquired other essential functions that have been retained by this physiologically-diverse molecule.

Tryptophan is the starting molecule for melatonin production in cell species. The sequence of the enzymatic steps that convert tryptophan to melatonin, however, varies among species. These steps include hydroxylation, decarboxylation, acetylation, and methylation. In some plant species, melatonin may not be the end product; in at least one variety of rice, melatonin can be hydroxylated at either 2, 4, or 6 position with 2-hydroxymelatonin possessing significant antioxidant activity, like melatonin itself. While the synthetic pathway of melatonin has changed throughout evolution and differs among plant and animal species, the structure of melatonin persists as originally designed in bacteria billions of years ago. It is pointed out, however, that what is known about melatonin synthesis has come primarily from mammals and the pathway in other vertebrates has been sparingly investigated. Moreover, the pathway of melatonin production in invertebrates remains to be examined.

AUTHOR CONTRIBUTIONS

RR initiated the review and checked all drafts of the report. DZ, YY, and YS worked together to write the initial drafts of the manuscript. QL and RS prepared figures for the article and read the final version. ZZ and RS participated in the discussion of the functional evolution of melatonin.

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Melatonin: Clinical Perspectives in Neurodegeneration

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Prevention of neurodegenerative diseases is presently a major goal for our Society and melatonin, an unusual phylogenetically conserved molecule present in all aerobic organisms, merits consideration in this respect. Melatonin combines both chronobiotic and cytoprotective properties. As a chronobiotic, melatonin can modify phase and amplitude of biological rhythms. As a cytoprotective molecule, melatonin reverses the low degree inflammatory damage seen in neurodegenerative disorders and aging. Low levels of melatonin in blood characterizes advancing age. In experimental models of Alzheimer's disease (AD) and Parkinson's disease (PD) the neurodegeneration observed is prevented by melatonin. Melatonin also increased removal of toxic proteins by the brain glymphatic system. A limited number of clinical trials endorse melatonin's potentiality in AD and PD, particularly at an early stage of disease. Calculations derived from animal studies indicate cytoprotective melatonin doses in the 40–100 mg/day range. Hence, controlled studies employing melatonin doses in this range are urgently needed. The off-label use of melatonin is discussed.

Keywords: aging, Alzheimer's disease, glymphatic system, melatonin, mild cognitive impairment, neurodegeneration, oxidative stress, Parkinson's disease

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INTRODUCTION

Symmetrical losses of neurons in the cognitive, motor, or sensory systems characterize neurodegenerative diseases showing prominent cognitive symptoms, like Alzheimer's disease (AD) and frontotemporal dementia, or predominantly motor symptoms like Parkinson's disease (PD), Huntington's disease or amyotrophic lateral sclerosis. Neuronal death in these entities can be ascribed to interrelated processes like free radical-mediated degeneration, mitochondrial dysfunction, low degree of inflammation and excitotoxicity (1, 2). Although the regular intake of antioxidants has been proposed for prevention of neurodegeneration, its effectiveness, however, has been questioned (3). In this context, the cytoprotection given by melatonin use deserves to be considered.

Melatonin, an unusual phylogenetically conserved compound present in all known aerobic phyla, has a promising significance as a cytoprotective molecule in addition to its chronobiotic properties (4). The pineal gland is the demonstrable source of melatonin in circulation, the decrease in plasma melatonin being one of the characteristics of advancing age in humans (5). The focus of this article is on the clinical use of melatonin in neurodegenerative diseases. The discussion of the basic biological data is restricted to its relevance for melatonin doses potentially employable in humans.

BASIC BIOLOGY OF MELATONIN

"Chronobiotics" are defined as drugs displaying the capacity to synchronize or to increase the amplitude of the circadian rhythms, melatonin being the prototype (6, 7). Light-dark variation of melatonin synthesis defines the essential role of melatonin as a chronobiotic (8). Melatonin "opens the doors of sleep" by inhibiting the propensity to wakefulness derived from the suprachiasmatic nuclei (SCN) in late evening (9, 10). On the other hand, melatonin is the chemical code of darkness, an information crucial to the neuroendocrine system (11).

Although in mammals the circulating melatonin derives almost exclusively from the pineal gland (5), the methoxyindole is synthesized locally in most cells, tissues, and organs (12). Indeed, there is now strong evidence that melatonin is produced in every animal cell that has mitochondria (13, 14), melatonin being involved, among other functions, in the elimination of free radicals and the regulation of immune response to achieve cytoprotection (15).

MT_1 and MT_2 receptors, all belonging to the superfamily of membrane receptors associated with G proteins (G-protein coupled receptors, GPCR) are involved in the chronobiotic action of melatonin (16). MT_1 and MT_2 receptors have been identified in the SCN, hippocampus, thalamus, retina, vestibular nuclei and cerebral and cerebellar cortex (17).

More recently, another member, GPR50, was included in the melatonin receptor subfamily showing high sequence homology with MT_1 and MT_2 (18). However, GPR50 does not bind melatonin or any other known ligand. Rather, they form homo- and heteromers between each other and with other GPCRs (19).

Melatonin is not only generated and metabolized in the mitochondria but it was recently claimed that the neuroprotective effects of melatonin on the brain injury induced by ischemia/reperfusion were mediated by MT_1 receptors located in the mitochondria but not in the membrane (20). This is remarkable because a GPCR like the MT_1 is known as a cell-surface receptor that transmits extracellular signals into the cell.

Melatonin, an amphiphilic substance, can penetrate cell membranes. In the cytoplasm melatonin interacts with calmodulin and tubulin (21). Melatonin also enters the cell nucleus where the receptor sites were supposed to belong to the orphan receptor superfamily RZR/ROR (15). However, RZR/ROR demonstrably does not bind melatonin. Rather, melatonin may act indirectly via this transcription factor, e.g., by affecting the circadian accessory oscillator component RORα through sirtuin-1 (SIRT-1) activation (22).

The cytoprotective activity of melatonin exceeds that mediated via receptors. The amounts of melatonin found in almost every cell are much higher than those in circulation (12). Although the capacity of mitochondria to synthesize melatonin is now confirmed, intracellular melatonin does not get the extracellular space. Indeed, the doses of melatonin needed to change intracellular melatonin concentration are much higher than those employed as a chronobiotic (23, 24).

In cell cultures, physiologically relevant effects of melatonin are revealed at doses in the range of 10^{-8} to 10^{-9} M, these concentrations being enough for almost complete or total receptor saturation (25, 26). However, most studies on neuroprotective and anti-inflammatory effects in animals employ pharmacological doses, which clearly exceed the saturation of the receptor.

The focus in this review is on melatonin effects on neurodegeneration in animal studies as related to the possible human doses to be employed. It must be noted that cell line studies regarding AD and melatonin have delineated important melatonin mediated mechanisms in the line of prevention against AD as well. A comprehensive review on melatonin activity to reverse disrupted signaling mechanisms in neurodegeneration, including proteostasis dysfunction, disruption of autophagic integrity, and anomalies in the insulin, Notch, and Wnt/β-catenin signaling pathways has just been published (27).

In a way largely independent of receptors melatonin has antioxidant and scavenging effects (28). Melatonin has intrinsic free radical scavenging activity as well as is metabolized to compounds that display a higher antioxidant capacity. In addition, melatonin inhibits the synthesis of prooxidant enzymes and facilitates that of antioxidant enzymes. Melatonin exceeds that capacity of vitamin C and E to protect from oxidative damage (29). Melatonin also exerts cytoprotection in ischemia (independently of free radical scavenging) presumably via mitochondrial membrane stabilization (24).

Immunomodulation by melatonin includes proinflammatory and anti-inflammatory effects (30–32). The anti-inflammatory actions are of great medical interest since they are found in high-grade inflammation like brain injury, ischemia/reperfusion or sepsis, as well as in low-grade inflammation like aging or neurodegenerative processes.

The anti-inflammatory properties of melatonin are exerted by inhibiting the binding of nuclear factor κB (NF κB) to DNA (thus decreasing the synthesis of proinflammatory signals), by inhibiting cyclooxygenase (Cox) (21) mainly Cox-2 (33), and by suppressing the expression of the inducible nitric oxide synthase (iNOS) (34). Other signaling pathways involved include prevention of inflammasome NLRP3 activation, upregulation of nuclear factor erythroid 2-related factor 2 and inhibition of toll-like receptor-4 activation and high-mobility group box-1 signaling. The upregulation of SIRT-1 by melatonin appears to be of major importance. Collectively, these effects of melatonin are reflected in reduced levels of proinflammatory cytokines and increased production of anti-inflammatory cytokines (31).

The γ-aminobutyric acid (GABA)-ergic system can be involved in the neuroprotection mediated by melatonin. Indeed, melatonin exerts anti-excitatory and sedative effects (35, 36) and information exists that melatonin gives protection to neurons from the toxicity of the amyloid-β (Aβ) peptide via activation of GABAergic receptors (37). The up-regulation of GABA activity by melatonin could not be blocked by the melatonin receptor antagonist luzindole but it was impaired by the benzodiazepine antagonist flumazenil, suggesting an allosteric modulation of $GABA_A$ receptors by melatonin (38).

Melatonin displays also anti-excitotoxic activity. For example, melatonin prevents neuronal death induced by kainate, an ionotropic glutamate receptor agonist (39), and its administration protects hippocampal CA1 neurons from

transient anterior ischemia (40), or from high doses of glucocorticoids (41). The lack of effects of luzindole or of the MT_2 antagonist 4-phenyl-2-propionamidotetralin (4-P-PDOT) excludes the participation of melatonin receptors in melatonin anti-excitotoxic activity (42).

In addition to the animal models of AD and PD that are discussed in the present paper, melatonin has been shown to reduce neuronal damage due to the toxicity of cadmium (43, 44), hyperbaric hyperoxia (45, 46), toxicity by δ-aminolevulinic acid (47), γ radiation (48), focal ischemia (49), brain trauma (50, 51), and that resultant from several neurotoxins (52).

MELATONIN ACTIVITY IN ANIMAL MODELS OF AD

The extracellular deposits of Aß-formed senile plaques and the intracellular accumulation of neurofibrillary tangles due to hyperphosphorylation of tau protein are the pathological signatures of AD (1, 2). Aß promotes neuronal degeneration in AD neurons that have become vulnerable to age-related increases of oxidative stress and altered cellular energy metabolism. Hyperphosphorylated tau protein promotes the assembly of microtubules and is an important factor in stabilizing microtubules (1, 2).

The 39–43 amino acid residue Aß derives from the amyloid precursor protein (APP). Melatonin interferes with the maturation of APP in several cell lines (53).

Table 1 summarizes the effect of melatonin in transgenic models of AD. The data indicate that melatonin modulates APP and Aß metabolism principally at the initial phases of the pathological process. From the doses of melatonin used in these different transgenic models, the human equivalent dose of melatonin for a 75 kg adult can be calculated by normalization of body surface area (54). Noteworthy, theoretical human equivalent doses calculated from **Table 1** results ranged from 2- to 3-orders of magnitude greater than those employed in humans. However, a note of caution must be made since these studies include changes in the expression of genes with mutations characteristic of the hereditary form of AD, only responsible for 5% of AD cases. Senescence-accelerated OXYS rats appears to be a suitable non-transgenic model of sporadic AD (which accounts for 95% of AD patients) as characterized by the progressive age-related aggregation of Aβ and hyperphosphorylation of τ protein as well as mitochondrial dysfunction, loss of synapses, neuronal death, and concomitant cognitive decline (72). Remarkable, a very low dose of melatonin (0.04 mg daily p.o.) was effective to prevent all these changes (73). Additional studies are needed to solve the dose incongruences observed.

How melatonin inhibits generation of Aβ remains undefined. Melatonin may interact with $Aß_{40}$ and $Aß_{42}$ inhibiting the formation of progressive β-sheet and/or amyloid fibrils (74, 75). Such interaction appears to be independent on melatonin antioxidant properties (74).

Via blockage of formation of secondary sheets, melatonin may facilitate the peptide clearance induced by proteolytic degradation. Since GSK−3 is a common signaling pathway increasing Aß generation and tau hyperphosphorylation, melatonin could regulate APP and tau processing via protein kinase (PK) C activation (76, 77) and inhibition of GSK-3 pathway (78). Free radical are involved in Aß-induced neurotoxicity and cell death, melatonin effectively protecting cells against oxidative damage *in vitro* (79, 80) and *in vivo* (81–83).

In N2a and SH-SY5Y neuroblastoma cells exposed to wortmannin (84), calyculin A (85, 86), or okadaic acid (87–89) melatonin efficiently attenuated tau hyperphosphorylation via protein kinases and phosphatases, as well as antagonizes the oxidative stress given by these agents (90, 91). Regulation of PK A (92), PK C (93), Ca^{2+}/calmodulin-dependent kinase II (94), and mitogen-activated protein kinase are other effects of melatonin unrelated to its antioxidant properties (95).

A crucial phenomenon for brain homeostasis is waste products' elimination by the glymphatic system. The term "glymphatic" describes active, lymphatic-like, water exchange movements in the brain extracellular space (ECS) driven by perivascular astrocytes, which contain aquaporin-4 (AQP4) located in their end feet (96). Since the elimination of Aβ peptide is strongly reduced in $AQP4^{-/-}$ mice (97), occurrence of an AQP4-driven glymphatic Aβ clearance seems feasible.

During sleep, the elimination of Aβ peptides increases considerably (98). Thus, the sleep disturbance found as a comorbidity in AD may contribute to the development and progression of the disease via a failure of Aβ clearance. Sleep deprivation disrupted apolipoprotein E clearance from brain ECS (99). That the glymphatic system participates in tau protein clearance was further indicated by the demonstration that AQP4 deficiency augmented the presence of extracellular tau and neuronal tangle formation in a murine model of traumatic brain injury (100).

An important recent observation by Pappolla et al. indicate that the administration of melatonin to AD transgenic mice augments the glymphatic clearance of Aβ (101). Relevant to this, melatonin is known to preserve slow wave sleep in patients (102). Indeed, glymphatic dysfunction has been related to various neurological disease in addition to AD, like stroke or traumatic brain injury (103).

The rise in the expression of proinflammatory cytokines triggered by microglial activation seems to play a role in the pathogenesis of AD (1, 2). Microglial release of proinflammatory cytokines induced by NF kB, Aß, and nitric oxide is effectively halted by melatonin (83). Binding of NF kB to DNA was also inhibited by melatonin (22, 31).

CLINICAL APPLICATION OF MELATONIN IN AD

Cerebrospinal fluid (CSF) concentration of melatonin decreases even at the preclinical stages of AD (104). Circulating melatonin correlates negatively with neuropsychological evaluation in mild cognitive impairment (MCI) and AD patients (105). The relative deficiency of melatonin could be the cause or a consequence of neurodegeneration. In any event, the loss of melatonin aggravates

TABLE 1 | Effect of melatonin on transgenic models of AD.

References	Design	Results	Melatonin human equivalent dose for a 75 kg adult
Matsubara et al. (55)	4-month-old APP 695 transgenic mice received 50 mg/kg of melatonin in drinking water for 8, 9.5, 11, and 15.5 months	The administration of melatonin partially inhibited the expected time-dependent elevation of β-amyloid, reduced abnormal nitration of proteins, and increased survival	300 mg/day
Feng et al. (56)	4-month-old APP 695 transgenic mice received 10 mg/kg of melatonin in drinking water for 4 months	Melatonin counteracted learning and memory impairment in transgenic mice, as shown by step-down and step-through passive avoidance tests. Additionally, the decrease in choline acetyltransferase activity in the frontal cortex and hippocampus of transgenic mice was prevented by melatonin	60 mg/day
Quinn et al. (57)	14-month-old transgenic (Tg 2576) mice received 3.6 mg/kg melatonin in drinking water for 4 months	There were no differences between untreated and melatonin-treated transgenic mice in cortical levels Aβ, nor in brain levels of lipid peroxidation product. Melatonin fails to produce antiamyloid or antioxidant effects when initiated after the age of amyloid plaque deposition	20 mg/day
Feng et al. (58)	4-month-old APP 695 transgenic mice received 10 mg/kg of melatonin in drinking water for 4 months	Melatonin prevented the increase of brain thiobarbituric acid reactive substances, the decrease in glutathione content, and the upregulation of the apoptotic-related factors in transgenic mice	60 mg/day
Garcia et al. (59)	5-month-old female transgenic (Tg2576) were exposed for 6 months to aluminum (1 mg/g) or melatonin (10 mg/kg/day in drinking water)	No effect of aluminum on general motor activity was found. A lower habituation pattern was observed in melatonin-treated animals. Aluminum-treated Tg2576 mice showed impaired learning, an effect unmodified by melatonin treatment	60 mg/day
Olcese et al. (60)	Melatonin (20 mg/kg in drinking water) was given to 2–2.5 month-old APP/PS1 transgenic mice for 5 months	Transgenic mice given melatonin were protected from cognitive impairment in working memory, spatial reference learning/memory, and basic mnemonic function. Immunoreactive Aβ deposition was reduced in hippocampus and entorhinal cortex of melatonin treated transgenic mice. Melatonin decreased tumor necrosis factor-α in hippocampus and normalized cortical mRNA expression of antioxidant enzymes	120 mg/day
Garcia et al. (61)	5-month-old female transgenic (Tg2576) were exposed for 6 months to aluminum (1 mg Al/g diet) or melatonin (10 mg/kg/day in drinking water)	The prooxidant effect of aluminum in the hippocampus was prevented by melatonin	60 mg/day
Spuch et al. (62)	9-month-old male APP/PS1 transgenic mice were used. The tacrine–melatonin hybrid (2 μl per mouse, 50 μg/ml) was stereotaxically injected in each lateral ventricle and the animals were killed 6 weeks later	The intracerebral administration of tacrine-melatonin hybrid decreased Aβ-induced cell death and amyloid burden in the brain parenchyma of APP/Ps1 mice. The reduction in Aβ pathology was accompanied by the recovery of cognitive function	–
Bedrosian et al. (63)	Melatonin (1 mg/kg) was given nightly for 4 week to 9-month-old transgenic amyloid precursor protein (APPSWE) mice	A temporal pattern of anxiety-like behavior emerged in elderly mice and in transgenic APP mice i.e., elevated locomotor activity relative to adult mice near the end of the dark phase, and time-dependent changes in basal forebrain acetylcholinesterase expression. Melatonin treatment did not affect the modifications found in elderly or transgenic mice	6 mg/day
Dragicevic et al. (64)	18 to 20-month-old APP/PS1 transgenic mice received 20 mg/kg melatonin in drinking water for 1 month	Melatonin treatment decreased mitochondrial Aβ levels in several brain regions. This was accompanied by a near complete restoration of mitochondrial respiratory rates, membrane potential, and ATP levels in isolated mitochondria from the hippocampus, cortex, or striatum	120 mg/day
Baño et al. (65)	3.5 to 5.5-month-old APP/PS1 double transgenic mouse were given melatonin (5 mg/kg) or ramelteon (2 mg/kg) in drinking water or in re-pelleted food, respectively, for 5.5 months	Many of the circadian and behavioral parameters measured, including hippocampal oxidative stress markers, were not significantly affected in transgenic mice. Whereas, melatonin maintained τ at 24 h for body temperature and locomotor activity, ramelteon treatment had no effect. Brain tissue analysis revealed a significant reduction in hippocampal protein oxidation in transgenic mice treated with melatonin or ramelteon	30 mg/day

(Continued)

TABLE 1 | Continued

References	Design	Results	Melatonin human equivalent dose for a 75 kg adult
Dragicevic et al. (66)	11 to 12-month-old APPsw mice received 100 mg/kg of melatonin for 1 month	Melatonin treatment yielded a near complete restoration of brain mitochondrial function in assays of respiratory rate, membrane potential, reactive oxygen species production, and ATP levels	600 mg/day
Garcia-Mesa et al. (67)	6-month-old 3xTg-AD mice received 10 mg/kg for 6 months. Physical exercise was implemented by free access to a running wheel in the housing cage	Both melatonin and physical exercise decreased soluble amyloid β oligomers, whereas only melatonin decreased hyperphosphorylated tau. Both treatments protected against cognitive impairment, brain oxidative stress, and a decrease in mitochondrial DNA. Only the combined treatment of physical exercise plus melatonin was effective against the decrease of mitochondrial complexes	60 mg/day
McKenna et al. (68)	50 mg/kg ramelteon in drinking water was given to B6C3-Tg (APPswe, PSEN1dE9) 85Dbo/J mice for 6 months	Absence of effect of ramelteon on cognitive performance of AD mice (water maze) or Aβ deposits in cerebral cortex or hippocampus	–
Di Paolo et al. (69)	5-month-old female transgenic (Tg2576) were exposed for 14 months to aluminum (1 mg Al/g diet) or melatonin (10 mg/kg/day in drinking water)	Melatonin improved learning and spatial memory in aluminum-exposed transgenic mice	60 mg/day
Gerenu et al. (70)	4-month-old double-transgenic female APP/PS1 mice were administered with a curcumin/melatonin hybrid (Z-CM-I-1) (50 mg/kg) by oral gavage. Animals were treated 5 times per week for 12 consecutive weeks	Z-CM-I-1 decreased the accumulation of Aβ in the hippocampus and cerebral cortex and reduced inflammatory responses and oxidative stress. Z-CM-I-1 also increased expression of synaptic marker proteins PSD95 and synaptophysin and of complexes I, II, and IV of the mitochondria electron transport chain	150 mg/day
Nie et al. (71)	10-month-old triple transgenic mice (3xTg-AD) received melatonin (10 mg/kg/day in drinking water) for 1 month	Melatonin ameliorated anxiety and depression-like behaviors of 3xTg-AD mice. Hippocampal glutathione S-transferase P 1 (an anxiety associated protein) and complexin-1 (a depression associated protein) were significantly modulated by melatonin	60 mg/day

The human equivalent dose of melatonin for a 75 kg adult is calculated by normalization of body surface area (54).

the disease and causes early circadian disturbance as shown by "sundowning" (106). Sundowning comprises late afternoon or evening symptoms such as agitation, wandering, disorganized thinking, perceptual and emotional disturbances and reductions in attention. Chronotherapeutic interventions, such as timed administration of melatonin and exposure to bright light, relieve sundowning and improved sleep in AD patients (107, 108).

The irregular sleep/wake found in AD is effectively treated by melatonin (**Table 2**). A significant decrease in sundowning and reduced variability of sleep onset time were found in 7 out of 10 dementia patients with sleep disorders treated with 3 mg melatonin at bedtime for 3 weeks (109).

In another study including 14 AD patients treated with 6–9 mg/day for a period of 2–3 years, sleep quality improved (110). Sundowning was no longer detectable except for 2 patients. We also observed improvement of cognitive performance and reduction of amnesia after melatonin treatment. In a case report of monozygotic twins with AD followed for 36 months we reported a better sleep and cognitive function in the twin receiving melatonin (111).

The effectiveness of melatonin to improve sleep and alleviate sundowning were reported in open-label and placebo-controlled studies in AD patients (107, 112, 113, 115–120). Negative results were also published in fully developed AD patients treated with melatonin (114, 121). Indeed, large interindividual differences in sleep and agitation are common among AD patients.

A review of published results on the use of melatonin in AD (122) yielded seven reports (5 open studies, 2 case reports) (N = 89 patients) that supported a possible efficacy of melatonin in improving sleep, decreasing sundowning and improving cognitive deterioration. In six double blind, randomized placebo-controlled trials (N = 210 patients) sleep quality increased, sundowning decreased, and cognitive performance improved in 4 studies (N = 143) whereas there was absence of significant effects in 2 studies (N = 67) (122). Two meta-analyses supported the view that melatonin therapy is effective in improving sleep in patients with dementia (123, 124). In addition, the melatonergic agent ramelteon was effective in treating delirium of elderly patients in intensive care units (125).

Whether melatonin has any value in the treatment of fully developed AD remains undefined. It should be noted that the heterogeneity in pathology of the group examined is probably very high at this stage of disease. Therefore, information obtained at an earlier phase could be more valuable.

Patients with MCI have a deficit in cognitive functions with preservation of daily activities. MCI is a clinically important stage

TABLE 2 | Studies including treatment of AD patients with melatonin.

Subjects	Design	Study's duration	Treatment	Measured	Results	References
10 demented patients	Open-label study	3 weeks	3 mg melatonin p.o./daily at bed time	Daily logs of sleep and wake quality completed by caretakers	7 out of 10 dementia patients having sleep disorders treated with melatonin showed a significant decrease in sundowning and reduced variability of sleep onset time	(109)
14 AD patients	Open-label study	22–35 months	6–9 mg melatonin p.o./daily at bed time	Daily logs of sleep and wake quality completed by caretakers. Neuro-psychological assessment	Sundowning was no longer detectable in 12 patients and persisted, although attenuated in 2 patients. A significant improvement of sleep quality was found. Lack of progression of the cognitive and behavioral signs of the disease during the time they received melatonin	(110)
Monozygotic twins with AD	Case report	36 months	One of the patients was treated with melatonin 9 mg p.o./daily at bed time	Neuro-psychological assessment. Neuroimaging	Sleep and cognitive function severely impaired in the twin not receiving melatonin as compared to the melatonin-treated twin	(111)
11 AD patients	Open-label study	3 weeks	3 mg melatonin p.o./daily at bed time	Daily logs of sleep and wake quality completed by the nurses	Significant decrease in agitated behaviors in all three shifts; significant decrease in daytime sleepiness	(112)
14 AD patients	Open-label, placebo-controlled trial	4 weeks	6 mg melatonin p.o./daily at bed time or placebo	Daily logs of sleep and wake quality completed by caretakers. Actigraphy	AD patients receiving melatonin showed a significantly reduced percentage of nighttime activity compared to a placebo group	(113)
25 AD patients	Randomized double blind placebo controlled cross over study	7 weeks	6 mg of slow release melatonin p.o. or placebo at bed time	Actigraphy	Melatonin had no effect on median total time asleep, number of awakenings or sleep efficiency	(114)
45 AD patients	Open-label study	4 months	6–9 mg melatonin p.o./daily at bed time	Daily logs of sleep and wake quality completed by caretakers. Neuro-psychological assessment	Melatonin improved sleep and suppressed sundowning, an effect seen regardless of the concomitant medication employed	(107)
157 AD patients	Randomized placebo-controlled clinical trial	2 months	2.5-mg slow-release melatonin, or 10-mg melatonin or placebo at bed time	Actigraphy. Caregiver ratings of sleep quality	Non-significant trends for increased nocturnal total sleep time and decreased wake after sleep onset were observed in the melatonin groups relative to placebo. On subjective measures, caregiver ratings of sleep quality showed a significant improvement in the 2.5-mg sustained-release melatonin group relative to placebo	(115)
20 AD patients	Double-blind, placebo-controlled study	4 weeks	Placebo or 3 mg melatonin p.o./daily at bed time	Actigraphy. Neuro-psychological assessment	Melatonin significantly prolonged the sleep time and decreased activity in the night. Cognitive function was improved by melatonin	(116)
7 AD patients	Open-label study	3 weeks	3 mg melatonin p.o./daily at bed time	Actigraphy. Neuro-psychological assessment	Complete remission of day night rhythm disturbances or sundowning was seen in 4 patients, with partial remission in other 2	(117)
17 AD patients	Randomizedplacebo-controlled study	2 weeks	3 mg melatonin p.o./daily at bed time (7 patients). Placebo (10 patients)	Actigraphy. Neuro-psychological assessment	In melatonin-treated group, actigraphic nocturnal activity and agitation showed significant reductions compared to baseline	(118)
68-year-old man with AD who developed rapid eye movement (REM) sleep behavior disorder	Case report	20 months	5–10 mg melatonin p.o./daily at bed time	Polysomnography	Melatonin was effective to suppress REM sleep behavior disorder	(119)

(Continued)

TABLE 2 | Continued

Subjects	Design	Study's duration	Treatment	Measured	Results	References
50 AD patients	Randomizedplacebo-controlled study	10 weeks	Morning light exposure (2,500 lux, 1 h) and 5 mg melatonin (n= 16) or placebo (n = 17) in the evening. Control subjects (n = 17) received usual indoor light (150–200 lux)	Nighttime sleep variables, day sleep time, day activity, day: night sleep ratio, and rest-activity parameters were determined using actigraphy	Light treatment alone did not improve nighttime sleep, daytime wake, or rest-activity rhythm. Light treatment plus melatonin increased daytime wake time and activity levels and strengthened the rest-activity rhythm	(120)
41 AD patients	Randomizedplacebo-controlled study	10 days	Melatonin (8.5 mg immediate release and 1.5 mg sustained release) (N = 24) or placebo (N = 17) administered at 10:00 p.m.	Actigraphy	There were no significant effects of melatonin, compared with placebo, on sleep, circadian rhythms, or agitation	(121)

to identify and treat people at risk (126) because the estimate of the annual rate of conversion of MCI to dementia can be as high as 10–15%, In fact, the degenerative process in the brain of AD begins 20–30 years before the clinical onset of the disease (127–131).

As shown in **Table 3**, data published from MCI patients consistently showed that melatonin administration improves cognitive performance and sleep quality. For example, we reported a significant improvement of cognitive and depressive symptoms and sleep quality in 35 patients with MCI treated for up to 2 years with 3–9 mg/day of melatonin as an adjuvant (130). Significantly lower scores in Beck Depression Inventory and better performance in neuropsychological tests and in sleep and wakefulness subjective assessment were documented in 61 outpatients diagnosed with MCI and receiving 3–24 mg of melatonin daily for 15–60 months (134). Collectively, the results of **Table 3** indicate that melatonin is an adjuvant drug useful for the treatment of MCI in a clinical setting.

The mechanisms that explain the therapeutic effect of melatonin in patients with MCI have not yet been defined. Promotion of slow-wave sleep in the elderly could be beneficial in MCI by increasing the functioning of the glymphatic system, or the secretion of growth hormone and neurotrophins, linked to the restorative phase of sleep.

The question of whether melatonin has a therapeutic value in the prevention or treatment of MCI deserves further analysis. Multicenter double-blind studies are needed to explore and further investigate the potential and utility of melatonin as a preventive drug against dementia. The doses of melatonin used should be re-evaluated in view of the equivalent human doses of melatonin derived from preclinical data, as indicated in **Table 1**. Unfortunately, of the 64 clinical trials related to melatonin in an initial state (recruitment and non-recruitment) listed in PubMed (ClinicalTrials.gov Search results 01/03/2019) none is directed to this query.

STUDIES ON MELATONIN ACTIVITY IN ANIMAL MODELS OF PD

The progressive degeneration of neurons containing dopamine (DA) in the substantia nigra pars compacta (SNpc) characterizes PD (143). Since Lewy bodies are found not only in DA neurons but also in noradrenergic neurons of the brainstem, in serotonergic neurons of the raphe nuclei and in specific cholinergic neurons, PD is seen as a progressive disease affecting a variety of neurotransmitter systems. This explains the number of non-motor symptoms in PD, such as genitourinary, gastrointestinal, respiratory and cardiovascular disorders, anosmia and neuropsychiatric, visual, and sleep-related disorders. In fact, the non-motor preclinical phase of PD can cover more than 20 years, the relevance of neuroprotection being evident in this respect (144).

The inflammatory signature found in the pathogenesis of PD includes microglial activation, astrogliosis and lymphocytic infiltration (145). Several inflammatory mediators, e.g., NF-κB,

TABLE 3 | Studies including treatment of MCI patients with melatonin.

Subjects	Design	Study's duration	Treatment	Measured	Results	References
10 patients with MCI	Double-blind, placebo-controlled, crossover study	10 days	6 mg melatonin p.o./daily at bed time	Actigraphy. Neuropsychological assessment	Melatonin enhanced the rest-activity rhythm and improved sleep quality. Total sleep time unaffected. The ability to remember previously learned items improved along with a significant reduction in depressed mood	(132)
26 individuals with age-related MCI	Double-blind, placebo-controlled pilot study	4 weeks	1 mg melatonin p.o. or placebo at bed time	Sleep questionnaire and a battery of cognitive tests at baseline and at 4 weeks	Melatonin administration improved reported morning "restedness" and sleep latency after nocturnal awakening. It also improved scores on the California Verbal Learning Test-interference subtest	(133)
354 individuals with age-related MCI	Randomized, double blind, placebo-controlled study	3 weeks	Prolonged release melatonin (Circadin, 2 mg) or placebo, 2 h before bedtime	Leeds Sleep Evaluation and Pittsburgh Sleep Questionnaires, Clinical Global Improvement scale score and quality of life	PR-melatonin resulted in significant and clinically meaningful improvements in sleep quality, morning alertness, sleep onset latency and quality of life	(134)
60 MCI outpatients	Open-label, retrospective study	9–24 months	35 patients received daily 3–9 mg of a fast-release melatonin preparation p.o. at bedtime. Melatonin was given in addition to the standard medication	Daily logs of sleep and wake quality. Initial and final neuropsychological assessment	Abnormally high Beck Depression Inventory scores decreased in melatonin-treated patients, concomitantly with an improvement in wakefulness and sleep quality. Patients treated with melatonin showed significantly better performance in neuropsychological assessment	(135)
189 individuals with age-related cognitive decay	Long-term, double-blind, placebo-controlled, 2 × 2 factorial randomized study	1–3.5 years	Long-term daily treatment with whole-day bright (1,000 lux) or dim (300 lux) light. Evening melatonin (2.5 mg) or placebo administration	Standardized scales for cognitive and non-cognitive symptoms, limitations of activities of daily living, and adverse effects assessed every 6 months	Light attenuated cognitive deterioration and ameliorated depressive symptoms. Melatonin shortened sleep onset latency and increased sleep duration but adversely affected scores for depression. The combined treatment of bright light plus melatonin showed the best effects	(108)
22 individuals with age-related cognitive decay	Prospective, randomized, double-blind, placebo-controlled, study	2 months	Participants received 2 months of melatonin (5 mg p.o. /day) and 2 months of placebo	Sleep disorders were evaluated with the Northside Hospital Sleep Medicine Institute (NHSMI) test. Behavioral disorders were evaluated with the Yesavage Geriatric Depression Scale and Goldberg Anxiety Scale	Melatonin treatment significantly improved sleep quality scores. Depression also improved significantly after melatonin administration	(136)

(Continued)

TABLE 3 | Continued

Subjects	Design	Study's duration	Treatment	Measured	Results	References
25 MCI outpatients	Randomized, double-blind, placebo-controlled study	12 weeks	11 patients received an oily emulsion of docosahexaenoic acid-phospholipids containing melatonin (10 mg) and tryptophan (190 mg)	Initial and final neuropsychological assessment of orientation and cognitive functions, short-term and long-term memory, attentional abilities, executive functions, visuo-constructional and visuo-spatial abilities, language, and mood	Older adults with MCI had significant improvements in several measures of cognitive function when supplemented with an oily emulsion of DHA-phospholipids containing melatonin and tryptophan for 12 weeks, compared with the placebo. The antioxidant capacity of erythrocytes and membrane lipid composition improved after treatment	(137, 138)
96 MCI outpatients	Open-label, retrospective study	15–60 months	61 patients received daily 3–24 mg of a fast-release melatonin preparation p.o. at bedtime. Melatonin was given in addition to the standard medication	Daily logs of sleep and wake quality. Initial and final neuropsychological assessment	Abnormally high Beck Depression Inventory scores decreased in melatonin-treated patients, concomitantly with an improvement in wakefulness and sleep quality. Patients treated with melatonin showed significantly better performance in neuropsychological assessment. Only 6 out of 61 patients treated with melatonin needed concomitant benzodiazepine treatment vs. 22 out of 35 MCI patients not receiving melatonin	(139)
80 patients diagnosed with mild to moderate AD, with and without insomnia comorbidity, and receiving standard therapy (acetylcholinesterase inhibitors with or without memantine)	Randomized, double-blind, parallel-group study	28 weeks	Patients were treated for 2 weeks with placebo and then randomized (1:1) to receive 2 mg of prolonged release melatonin or placebo nightly for 24 weeks, followed by 2 weeks placebo	The AD Assessment Scale-Cognition (ADAS-Cog), Instrumental Activities of Daily Living (IADL), Mini-Mental State Examination (MMSE), sleep, as assessed by the Pittsburgh Sleep Quality Index (PSQI) and a daily sleep diary, and safety parameters were measured	Patients treated with melatonin had significantly better cognitive performance than those treated with placebo. Sleep efficiency, as measured by the PSQI, component 4, was also better. Differences were more significant at longer treatment duration	(140)
142 patients meeting DSM-IV-TR criteria for major depression disorder were enrolled	Double-blind, placebo-controlled, randomized trial	6 weeks	Combination treatment: (buspirone 15 mg with melatonin- 3 mg) vs. buspirone 15 mgmonotherapy, vs. placebo	Clinical global impression of severity (CGI-S) and improvement (CGI-I), the QIDS-SR16, and the Hamilton rating scale for anxiety (Ham-A) at the baseline, week 2, week 4, and week 6 endpoint	Treatment responders improved significantly more on the total CPFQ than non-responders regardless of treatment assignment. The cognitive dimension of the CPFQ score favored the combination treatment over the other two groups	(141)
139 patients older than 65 year. of age scheduled for hip arthroplasty	Prospective cohort study	7 days	Patients were randomized to receive 1 mg oral melatonin or placebo daily 1 h before bedtime 1 day before surgery and for another 5 consecutive days post-operatively	Subject assessment, including Mini-Mental State Examination (MMSE) score, subjective sleep quality, general well-being, post-operative fatigue, and visual analog scale for pain were evaluated pre-operatively and at days 1, 3, 5, and 7 after surgery	The MMSE score in the control group decreased significantly after surgery. The MMSE score in the melatonin group remained unchanged during the 7 days of monitoring. In addition, significant post-operative impairments of subjective sleep quality, general well-being, and fatigue were found in the control group when compared with the melatonin group	(142)

interleukin (IL)-1, IL-6, Cox-2, tumor necrosis factor-α, iNOS, and interferon-γ are produced by glial cells (2).

PD and other Lewy body diseases are characterized by the aggregation of fibrillar α-synuclein (146). Mitochondrial dysfunction plays a role in this process since the folding and aggregation of proteins are promoted by free radicals (147, 148).

To develop animal models of altered brain DA function, 6-hydroxydopamine (6-OHDA), or the neurotoxin 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), were injected into the nigrostriatal pathway of the rat (149).

Because of its potentiality to cause the disease in humans and in subhuman primates, MPTP is preferred among other neurotoxins to emulate parkinsonism in animal models. MPTP is selectively taken up by astrocytes and is metabolized into methyl 1-4 phenyl pyridinium (MPP^+), this cation causing increased production of free radicals, depletion of ATP, and apoptosis. MPTP toxicity is selective to SNpc neurons and induced loss of striatal spines in non-human primates (150). Such striatal spine loss is a consistent neuropathologic finding in post-mortem PD human brains. Although the MPTP-treated monkey is considered the best experimental model of PD, a major drawback is the consistent lack of other neuronal loss besides the nigrostriatal dopaminergic system (151).

In **Table 4** the *in vivo* effects of melatonin in several experimental models of PD are shown. Most experiments support the role of melatonin in prevention and treatment of experimental PD. As in the case of **Table 1**, the human equivalent doses of melatonin for a 75 kg adult, calculated by normalization of body surface area (54), are quoted for the sake of comparison with those employed in PD patients. Again, theoretical human equivalent doses derived from animal data are considerably greater than those employed in humans.

Most of the studies summarized in **Table 4** include pretreatment with the methoxyindole and therefore they rely on the neuroprotective effect of melatonin preventing the death of dopaminergic neurons and consequently motor dysfunction (**Table 4**). In addition, some studies failed to observe motor benefits of melatonin treatment in animal models of PD regardless of protection of neurodegeneration. For example, Bassani et al. reported that the melatonin post-treatment for 28 days preserved tyrosine hydroxylase positive neurons and DA levels of rotenone-lesioned rats and improved the depressive-like behavior in the absence of significant improvement of motor deficit (171).

In PD patients, administration of 3 mg of melatonin for 4 weeks improved the quality of sleep but did not affect motor symptoms (179). Moreover, by evaluating the effects of slow release melatonin preparation via intracerebroventricular implants in rats injected with 6-OHDA or MPTP, Willis and Armstrong (180) reported that melatonin indeed deteriorated motor performance. Remarkably, other studies reported an enhancement of motor deficit in animal models of PD after the administration of the antagonist of melatonin receptors ML-23 (181). The beneficial effect of melatonin antagonism on motor symptoms of PD could be explained by inhibition of DA release by the methoxyindole (182).

Regardless of these discrepancies, melatonin preventing activity on PD-related neurodegeneration is generally accepted (183–185). For example, melatonin inhibits α-synuclein assembly and attenuated kainic acid-induced neurotoxicity (186) and arsenite-induced apoptosis (187). Melatonin also impaired the augmented expression of α-synuclein in DA containing neurons following amphetamine administration (188, 189). Melatonin blocked α-synuclein fibril formation and destabilized preformed fibrils by inhibiting protofibril formation and secondary structure transitions and by reducing α-synuclein cytotoxicity (169, 190).

An insufficient clearance by the autophagic–lysosomal network (146) can explain the accumulation and spread of oligomeric forms of neurotoxic α-synuclein. In addition, other clearance pathways are compromised, like the ubiquitin–proteasome system, the autophagy mediated by chaperone, extracellular clearance by proteases, or entrance into the general circulation via the glymphatic system (146).

As above mentioned, the elimination of waste products by the glymphatic system considerably contributes to recovery processes in the brain. The role of AQ4 water channels in the glymphatic system seems to be crucial and, remarkably, AQ4 expression is severely disrupted in PD brains (191). It may explain why the CSF α-synuclein levels inversely correlate with symptoms in PD patients (192). The association of loss of sleep with impairment of the glymphatic clearance is important in the case of PD because rapid eye movement sleep behavior disorder (RBD) is a prodrome of PD. Melatonin administration to animals augments glymphatic clearance (101) as well as preserved sleep in patients. Curiously, melatonin was not listed in the myriad of drugs affecting anomalous protein clearance in the brain (146).

Symptomatically, an effective treatment for PD is the supplementation of DA in its precursor form L-dihydroxyphenylalanine (L-DOPA) that crosses the blood brain barrier. However, long-term administration of -L-DOPA leads to motor side effects like dyskinesias (193, 194). Moreover, administration of L-DOPA in high doses leads to production of neurotoxic molecules like 6-OHDA. Therefore, efforts to reduce the intake or to compensate for the side effects of L-DOPA are in the vogue. In MPTP-treated mice, melatonin, but not L-DOPA, restored striatal spine density, supporting the application of melatonin as an adjuvant to L-DOPA therapy in PD (173).

CLINICAL USE OF MELATONIN IN PD

Approximately 3/4 of the dopaminergic cells in the SNpc need to be lost to uncover motor symptomatology in PD. However, non-motor symptoms like hyposmia, depression, or RBD (characterized by the occurrence of vivid, intense, and violent movements during REM sleep) precede the onset of PD for years and are index of worse prognosis (144). Indeed, up to 65% of patients showing RBD developed PD 10–13 years later (195).

Table 5 summarizes the clinical studies reporting melatonin use in PD. Daily administration of 3–12 mg of melatonin at bedtime is effective in the treatment of RBD (198–206). Polysomnography (PSG) in RBD patients treated with melatonin

TABLE 4 | *In vivo* effect of melatonin in animal models of PD.

References	Design	Results	Melatonin human equivalent dose for a 75 kg adult
Burton et al. (152)	Wistar rats receiving 6-OHDA injection into the SNc were treated with melatonin (1 and 10 mg/kg, i.p.)	Melatonin treatment inhibited apomorphine-induced rotational behavior	12 and 120 mg
Acuña-Castroviejo et al. (153)	C57BL/6 mice receiving an injection of MPP^+ were treated with melatonin (10 mg/kg, i.p.)	Melatonin treatment prevented MPTP-induced lipid peroxidation and TH-positive neuronal loss in striatum	60 mg
Jin et al. (154)	Sprague-Dawley rats receiving an injection of MPP^+ into the SNc were treated with melatonin (10 mg/kg, i.p.)	Melatonin treatment reduced lipid peroxidation and protected against DA neuronal loss induced by MPP^+	120 mg
Joo et al. (155)	Sprague-Dawley rats receiving 6-OHDA injections into the striatum were administered with melatonin (3 and 10 mg/kg, i.p.)	Melatonin treatment counteracted the 6-OHDA-induced changes in striatal DA synthesis and levels	36 and 120 mg
Kim et al. (156)	Sprague-Dawley rats receiving 6-OHDA injections into the striatum were treated with melatonin (3 or 10 mg/kg, i.p.)	Melatonin treatment reduced motor deficit and protected against 6-OHDA-induced loss of dopaminergic neurons	36 and 120 mg
Dabbeni-Sala et al. (157)	Sprague-Dawley rats receiving 6-OHDA injection into the SNc were treated with melatonin (50 ± 7.5 μg/h, s.c.)	Melatonin treatment prevented apomorphine-induced rotational behavior and mitochondrial damage	15 mg
Aguiar et al. (158)	Wistar rats receiving 6-OHDA injections into the striatum were administered with melatonin (2, 5, 10, and 25 mg/kg, i.p.)	Melatonin treatment prevented apomorphine-induced rotational behavior and depletion of striatal DA and serotonin levels	24–300 mg
Chen et al. (159)	Wistar rats receiving an injection of MPP^+ were treated with melatonin (10 mg/kg, i.p.)	Melatonin decreased MPP^+-induced toxicity and recovered GSH levels	120 mg
Khaldy et al. (160)	C57BL/6 mice receiving an injection of MPP^+ were treated with melatonin (5 or 10 mg/kg i.p.)	Melatonin protected damage of mitochondrial complex I activity in nigrostriatal neurons	30 and 60 mg
Sharma et al. (161)	Sprague-Dawley rats receiving 6-OHDA injections into the striatum were treated with melatonin (4 μg/mL) in drinking water	Melatonin normalized motor deficits and augmented TH immunoreactivity	6 mg
Singh et al. (162)	Sprague-Dawley rats receiving 6-OHDA injections into the striatum were treated with melatonin (0.5 mg/kg, i.p.)	Melatonin treatment prevented apomorphine-induced rotational behavior	6 mg
Saravanan et al. (163)	Sprague-Dawley rats were injected with rotenone into the SN. Melatonin (10, 20, or 30 mg/kg) was administrated i.p.	Melatonin reduced the levels of hydroxyl radicals in mitochondria and protected GSH levels and antioxidant enzymes activities in SN	120, 240, and 360 mg
Huang et al. (164)	Wistar rats receiving an injection of MPP^+ were treated with melatonin (10 mg/kg, i.p.)	Melatonin protected DA neurons from apoptosis induced by MPP^+	120 mg
Tapias et al. (165)	C57BL/6 mice received a single injection of MPTP. Melatonin (20 mg/kg) was given s.c.	Melatonin treatment prevented the MPTP-induced mitochondrial increase of NO, inhibited lipid peroxidation and protected complex I activity in striatum and SNc	120 mg
Patki et al. (166)	C57BL/6 mice received MPTP i.p. injections for 5 weeks. Melatonin (5 mg/kg) was administered i.p.	Melatonin protected against MPTP-induced DA neurons loss and locomotor activity deficit, and recovered mitochondrial respiration, ATP production, and antioxidant enzyme levels in SNc	30 mg
Singhal et al. (167)	Swiss mice treated with maneb plus paraquat received melatonin (30 mg/kg/day, i.p.)	Melatonin treatment protected lipid peroxidation and TH-positive neurons degeneration and prevented apoptosis	180 mg
Gutierrez-Valdez et al. (168)	Wistar rats receiving 6-OHDA injections into the medial forebrain bundle were treated with melatonin (10 mg/kg, p.o.)	Melatonin treatment improved motor performance without causing dyskinesia. Melatonin also protected TH-positive neurons and neuronal ultrastructure of striatum	120 mg
Brito-Armas et al. (169)	Sprague-Dawley rats injected with lentiviral vectors encoding mutant human α-synuclein in the SNc received melatonin treatment (10 mg/kg/day, i.p.)	Melatonin treatment prevented the loss of TH-positive neurons	120 mg

(Continued)

TABLE 4 | Continued

References	Design	Results	Melatonin human equivalent dose for a 75 kg adult
Zaitone et al. (170)	Swiss mice received 4 injections of MPTP. Melatonin was given p.o. (5 or 10 mg/kg/day)	Melatonin treatment recovered motor performance, striatal DA level, GSH, and antioxidant enzyme activities, and reduced lipid peroxidation. Melatonin improved the motor response to L-DOPA	30 and 60 mg
Bassani et al. (171)	Wistar rats were i.p. injected with rotenone. Melatonin (10 mg/kg) was administrated i.p.	Melatonin treatment protect TH-positive neurons in SNc and striatal levels of DA	120 mg
Yildirim et al. (172)	Wistar rats receiving 6-OHDA injections into the medial forebrain bundle were treated with melatonin (10 mg/kg, i.p.)	Melatonin prevented oxidative damage and apoptosis of dopaminergic neurons	120 mg
Naskar et al. (173)	BALB/c mice treated with MPTP received melatonin (10, 20, or 30 mg/kg, i.p.)	Melatonin protected against MPTP-induced TH-positive neurons loss in SNc and enhanced the therapeutic efect of L-DOPA	60, 120, and 180 mg
Ozsoy et al. (174)	Wistar rats receiving 6-OHDA injections into the medial forebrain bundle were treated with melatonin (10 mg/kg/day, i.p.)	Melatonin treatment protected DA neurons against changes in antioxidant enzyme activities and lipid peroxidation	120 mg
Carriere et al. (175)	Sprague Dawley rats were injected with rotenone. Melatonin (4.0 μg/mL) was given in drinking water	Melatonin treatment protected motor deficit and loss of TH-positive neurons in striatum and SNc after rotenone	6 mg
Li et al. (176)	Wistar rats were injected with 6-OHDA in the SNc and ventral tegmental area and received i.p. injections of melatonin (5 mg/kg)	Melatonin prevented DA neuronal damage	60 mg
Lopez et al. (177)	C57BL/6 mice receiving MPTP were administered melatonin (10 mg/kg s.c.)	Melatonin administration prevented the disruption of mitochondrial oxygen consumption, increased NOS activity and reduced locomotor activity induced by MPTP, independently of its anti-inflammatory properties	60 mg
Paul et al. (178)	Wistar rats injected with homocysteine in the SNc received melatonin treatment (10, 20, or 30 mg/kg/day, i.p.)	Treatment of melatonin protected against nigral DA loss and improved mitochondrial complex-I activity in SN	120, 240, and 360 mg

The human equivalent dose of melatonin for a 75 kg adult are calculated by normalization of body surface area (54).

showed significant decreases in number of R epochs without atonia and in movement time during REM sleep, contrasting with the persistence of muscle tone in R sleep seen with patients treated with clonazepam. Based on these data, a clinical consensus recommended melatonin use in RBD at Level B (207).

Another consensus has claimed for trials with neuroprotective agents in RBD based on the high conversion rate from idiopathic RBD to PD (195). Indeed, the conversion rate to synucleinopathy in clonazepam-treated RBD patients is high (208, 209). Although no comparable data are available yet for melatonin-treated RBD patients, a recent observation by Kunz and Bes deserves to be considered (205). The investigators reported the increase in DA transporter density (as assessed by DA transporter scintigraphy) over successive years in a 72-year-old RBD male patient treated with 2 mg of slow release melatonin daily. After 6 months of gradual improvement, clinical and PSG signs of RBD disappeared. Whereas, the scan prior to melatonin treatment had clear signs of PD, the scan recorded 2 years later was considered borderline, with absence of any sign of PD 4 years after the first scan. The results were interpreted as a possible neuroprotective role for melatonin in synucleinopathy (205).

A phase advance in nocturnal melatonin secretion was reported in L-DOPA-treated parkinsonian patients (210, 211). L-DOPA-treated patients exhibited an increase in daytime melatonin secretion perhaps as an adaptive response to neurodegeneration (211). In a study aiming to examine circadian dysfunction as a cause for excessive sleepiness in PD, blunted circadian rhythms of melatonin were reported (212). The amplitude of the melatonin rhythm decreased in PD patients, mainly in those depicting excessive daytime sleepiness. Thus, a chronobiological approach to improve circadian function, such as timed exposure to melatonin and bright light, could serve as an adjuvant therapy for the non-motor manifestations of PD.

An association between motor fluctuations in PD and diurnal variation in circulating melatonin levels was postulated via possible interactions of melatonin with monoamines (DA, serotonin) in the striatal complex (213). Nearly half of the patients with PD showed L-DOPA-related motor complications after 5 years of treatment. In view of the results obtained in experimental parkinsonism discussed above, the use of melatonin as an adjuvant to decrease the therapeutic dosage of L-DOPA in PD deserves to be considered (214).

Wearing-off episodes in PD could be related to loss of the inhibitory motor effect of melatonin, since stimulation of globus pallidus improved motor symptoms and complications in patients with PD as well as inhibited the increase in

TABLE 5 | Studies including treatment of PD and RBD patients with melatonin.

Subjects	Design	Study's duration	Treatment	Measured	Results	References
40 PD patients	Open-label, placebo-controlled trial	2 weeks	5–50 mg melatonin p.o./daily at bed time. All subjects were taking stable doses of antiparkinsonian medications	Actigraphy	Relative to placebo, treatment with 50 mg of melatonin significantly increased night time sleep, as revealed by actigraphy. As compared to 50 mg or placebo, administration of 5 mg of melatonin was associated with significant improvement of sleep in the subjective reports	(196)
18 PD patients	Open-label, placebo-controlled trial	4 weeks	3 mg melatonin p.o./daily at bed time	Polysomnography (PSG). Subjective evaluation by the Pittsburgh Sleep Quality Index and Epworth Sleepiness Scale	On initial assessment, 14 patients showed poor quality sleep EDS. Increased sleep latency (50%), REM sleep without atonia (66%), and reduced sleep efficiency (72%) were found in PSG. Melatonin significantly improved subjective quality of sleep. Motor dysfunction was not improved using melatonin	(179)
38 patients with PD without dementia and with complaints on sleep disorders	Open-label trial	6 weeks	Group 1 ($n = 20$) received 3 mg melatonin in addition to the previous dopaminergic group 2 ($n = 18$) received clonazepam 2 mg at night	Polysomnography (PSG) at baseline and at the end of the trial. Subjective evaluation by the PD sleep scale (PDSS) and the Epworth Sleepiness Scale (ESS). Neuropsychological testing using MMSE, five-word test, digit span and the Hamilton scale	Compared to baseline, melatonin and clonazepam reduced sleep disorders in patients. The daytime sleepiness (ESS) was significantly increased in the clonazepam group. Patients treated with melatonin had better scores on the MMSE, five-word test, Hamilton scale at the end of the study period as compared with the clonazepam group. Changes in total point scores on the PSG at the end of week 6 were in favor of the group treated with melatonin	(197)
1 RBD patient	Case report	5 months	3 mg melatonin p.o./daily at bed time	Actigraphy, PSG	Significant reduction of motor activity during sleep, as measured by actigraphy. After 2 months' treatment, PSG showed no major changes except an increase of REM sleep	(198)
6 consecutive RBD patients	Open-label prospective case series	6 weeks	3 mg melatonin p.o./daily at bed time	PSG	Significant PSG improvement in 5 patients within a week which extended beyond the end of treatment for weeks or months	(199)
14 RBD patients	Open-label prospective case series	Variable	3–9 mg melatonin p.o./daily at bed time	PSG	Thirteen patients and their partners noticed a suppressing effect on problem sleep behaviors after melatonin administration. % tonic REM activity in PSG findings was decreased after melatonin administration. Melatonin concentrations in 10 RBD patients were under 30 pg/mL at maximal values, their mean 33.5 pg/mL RBD patients with low melatonin secretion tended to respond to melatonin therapy	(200)
14 RBD patients	Retrospective case series	14 months	3–12 mg melatonin p.o./daily at bed time	PSG	8 patients experienced continued benefit with melatonin beyond 12 months of therapy	(201)
45 RBD patients	Retrospective case series		All initially treated with clonazepam. When melatonin was used, it was given at a 10 mg p.o./daily at bed time		21 patients continued to take clonazepam, 8 used another medication, and 4 required a combination of medications to control symptoms adequately	(202)

(Continued)

TABLE 5 | Continued

Subjects	Design	Study's duration	Treatment	Measured	Results	References
25 RBD patients	Retrospective case series	27–53 months	6 mg melatonin p.o./daily at bed time		As compared to clonazepam-treated RBD patients ($n = 18$) patients receiving melatonin reported significantly reduced injuries and fewer adverse effects	(203)
8 RBD patients	Double blind, placebo-controlled trial	4 weeks	3 mg melatonin p.o./daily at bed time.	PSG	Reduced number of 30-s epochs of REM without atonia and reduced frequency of RBD episodes	(204)
1 RBD patient	Case report	5 years	2 mg prolonged release melatonin p.o./daily at bed time	PSG and DA transporter scintigraphy (DaTSCAN)	A then 72-year-old man was clinically suspected to suffer from PD in 2011. DaTSCAN revealed reduced DA transporter density and PSG confirmed the diagnosis of RBD. After 6 months of melatonin treatment, clinical signs of RBD were absent. Control PSG in 2014 confirmed normalized REM sleep with atonia. Additional DaTSCANs were performed in 2013 and 2015 indicated normalization of DA transporter density	(205)
4 RBD patients with concomitant obstructive sleep apnea	Open label	4 weeks	2 mg prolonged release melatonin p.o./daily at bed time	PSG	Treatment led to a relevant clinical improvement of RBD symptoms in all patients, so far untreated for the sleep related breathing disorder. REM without atonia incidence was high probably because of the untreated comorbid condition	(206)

daytime plasma melatonin levels found (215). Relevant to the subject of the present review, genetic susceptibility and lifestyle factors (e.g., smoking) have been entertained to explain the epidemiological that longer years of working night shifts are associated with reduced risk of PD and decreased melatonin levels (216).

Patients with PD showed decreased melatonin MT_1 and MT_2 receptor density in amygdala and substantia nigra (217). Supporting that a disrupted melatonergic system could be involved in the altered sleep/wake cycle seen in PD, an actigraphic study undertaken in 40 PD patients indicated that melatonin (50 mg/day at bedtime) increased nighttime sleep. Those patients taking 5 mg of melatonin only reported a significant improvement of subjectively evaluated sleep (196).

In another study, 18 PD patients were randomized after performing a basal PSG to receive melatonin (3 mg) or placebo 1 h before bedtime for 4 weeks (179). Although melatonin significantly improved the subjective quality of sleep, the motor dysfunction was not improved (179).

An important experimental study carried out in the MPTP monkey model of PD evaluated the effects of melatonin and L-DOPA on sleep disorders as monitored by PSG (218). The combined treatment of melatonin and L-DOPA significantly curtailed sleep fragmentation at night and sleep episodes during the day seen in MPTP-treated monkeys, thus indicating that melatonin treatment may have the therapeutic potential to treat sleep disorder in PD patients.

Exposure to 1–1.5 h of light (1,000–1,500 lux) prior to bedtime reduced bradykinesia and rigidity in PD patients, as well as agitation and psychiatric side effects (219). The authors concluded that suppressing melatonin secretion with bright light may have a therapeutic value for treating the symptoms of PD (220). However, suppression of melatonin secretion may not be the likely mechanism by which artificial light exerts its therapeutic effect, as shown in depressive patients subjected to phototherapy (221). In any event, the circadian system is considered a novel diagnostic and therapeutic target in PD (212, 222).

Figure 1 summarizes that different mechanisms by which melatonin may halt AD and PD progression. Depicted intersections in the Figure represent the multiple effects of melatonin and the different degree of overlap (interrelations and mutual influences) discussed in the text.

CONCLUSIONS

Because of both hypnotic and chronobiotic properties, the use of melatonin has been recommended for treatment of insomnia (223, 224). Several meta-analyses support such therapeutic role (225–227). A consensus of the British Association for Psychopharmacology on evidence-based treatment of insomnia concluded that melatonin is the first choice treatment when a hypnotic is indicated in patients over 55 years (228).

As discussed in this article, studies using 2–5 mg melatonin/day are unsuitable to give appropriate comparison with data on neurodegeneration protection derived from animal

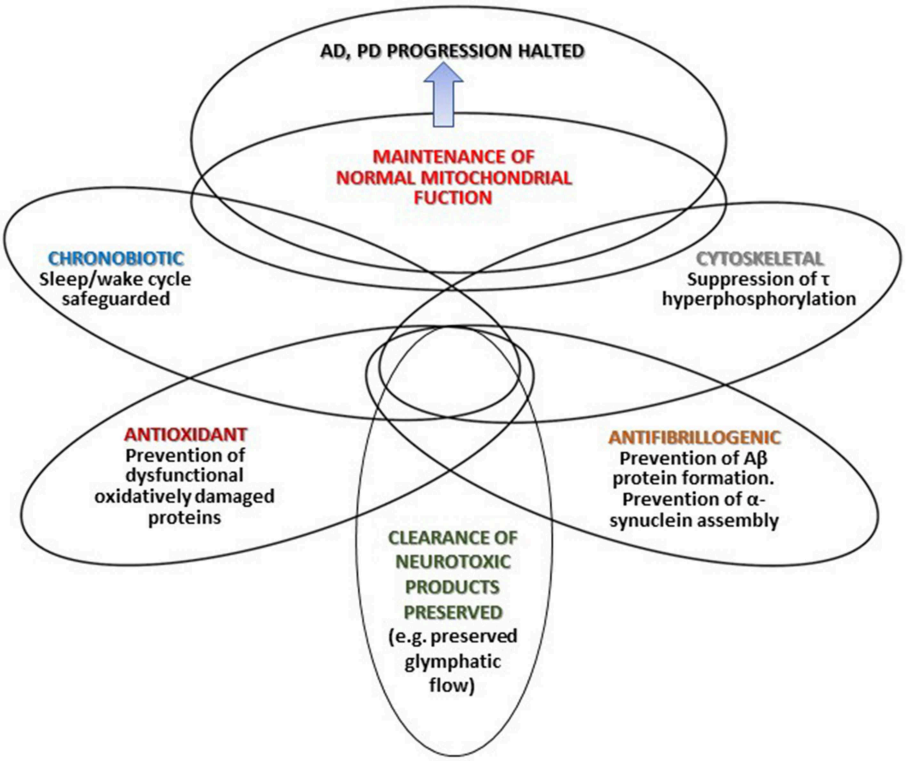

FIGURE 1 | The different mechanisms through which melatonin may halt AD and PD progression.

studies. Melatonin is remarkably atoxic and its safety is very high. Lethal dose 50 (LD50) for intraperitoneal melatonin injection was 1,131 mg/kg for mice and 1,168 mg/kg for rats. However, LD50 could not be constructed after oral administration of up to 3,200 mg of melatonin/kg in rats, or after subcutaneous injection of up to 1,600 mg/kg in rats and mice (229). In humans, melatonin has a high safety profile and it is usually remarkably well-tolerated (**Table 6**). Presently, the only option for the incumbent physician interested in the use of melatonin as a cytoprotective is the off-label indication of the drug.

Off-label drugs are defined as drug uses that not included in the indications or dosage regimens listed by the administrative body that registers, controls, and monitors medicines authorized, e.g., the Food and Drug Administration in USA (241). Off label drug use is common in intensive care unit, pediatrics, psychiatry and oncology (242–245). In general, no law prohibits off-label drug use and prescribing off-label is legally accepted in most legislations (246).

In Argentina, the National Administration for Medicaments, Food and Medical Technology (ANMAT) approved melatonin (3 mg capsules or tablets) as an over-the-counter medication in 1995. In 2017 ANMAT authorized a prolonged release preparation of 2 mg melatonin (Circadin^{R}) as a prescription drug. Although ANMAT cannot authorize the use of a medication for an indication not listed in the package leaflet, it does not mean that the indication of a medication for other clinical situations is prohibited. In accordance to ANMAT, The off-label prescriptions are "the sole responsibility of the attending physician, who performs them in the full exercise of their professional activity, based on their experience and the available scientific knowledge, motivated by the need to provide an answer to health problems for which there are no standards of treatment or that, in case of existing, they are very difficult to access."

In many countries, melatonin is used as a food supplement or dietetic products. Indeed, the European Food Safety Authority

TABLE 6 | Safety for off label prescription of melatonin.

Clinical condition	Melatonin dose	References
Dermal hyperpigmentation	1 g/day p.o. for 1 month	(230)
Parkinson's disease	0.25 and 1.25 mg/kg i.v.	(231)
Amyotrophic lateral sclerosis	60 mg/day p.o. for 13 months	(232)
Amyotrophic lateral sclerosis	300 mg/day, rectal for 2 years	(233)
Muscular dystrophy	70 mg/day for 9 months	(234)
Multiple sclerosis	50–300 mg/day p.o. for 4 years	(235)
Liver surgery	50 mg/kg	(236)
Healthy individuals	80 mg/h for 4 h	(237)
Healthy women	300 mg/day for 4 months	(238)
Dose escalation in healthy individuals	10–100 mg p.o.	(239)
Dose escalation in healthy individuals	10–100 mg p.o.	(240)

(EFSA) has endorsed the health claim that melatonin reduces sleep onset latency (247, 248). Thus, melatonin, melatonin-rich food and bioextracts could now be developed.

Overexpression of melatonin in plants facilitates the germination of seeds and protects plants from abiotic and biotic stress (249–251) and potentially genetically manipulated plants may have use in human nutrition. In parallel, toxicity of long-term melatonin use must be evaluated.

In conclusion, from animal studies several potentially useful effects of melatonin, like those in neurodegenerative disorders, need high doses of melatonin to become apparent. Regardless of the amount of experimental data gathered as far as how melatonin acts in animal and cell models, in most cases it is not known whether it works as a chronobiotic drug, as an endogenous antioxidant or as an immunomodulatory compound. This is an important caveat deserving consideration (252).

Although melatonin is remarkably atoxic and its safety is very high in adults, caution must be exerted with melatonin use in children taking in consideration that melatonin is known to inhibit LH secretion from neonatal pituitary gonadotrophs in the rat (253). Even if similar effect has not yet been documented in human, it cannot be excluded that treatment with high doses of exogenous melatonin could influence the development of the reproductive system.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Melatonin: Countering Chaotic Time Cues

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Last year melatonin was 60 years old, or at least its discovery was 60 years ago. The molecule itself may well be almost as old as life itself. So it is time to take yet another perspective on our understanding of its functions, effects and clinical uses. This is not a formal review—there is already a multitude of systematic reviews, narrative reviews, meta-analyses and even reviews of reviews. In view of the extraordinary variety of effects attributed to melatonin in the last 25 years, it is more of an attempt to sort out some areas where a consensus opinion exists, and where placebo controlled, randomized, clinical trials have confirmed early observations on therapeutic uses. The current upsurge of concern about the multiple health problems associated with disturbed circadian rhythms has generated interest in related therapeutic interventions, of which melatonin is one. The present text will consider the physiological role of endogenous melatonin, and the mostly pharmacological effects of exogenous treatment, on the assumption that normal circulating concentrations represent endogenous pineal production. It will concentrate mainly on the most researched, and accepted area of therapeutic use and potential use of melatonin—its undoubted ability to realign circadian rhythms and sleep—since this is the author's bias. It will touch briefly upon some other systems with prominent rhythmic attributes including certain cancers, the cardiovascular system, the entero-insular axis and metabolism together with the use of melatonin to assess circadian status. Many of the ills of the developed world relate to deranged rhythms—and everything is rhythmic unless proved otherwise.

Keywords: melatonin, circadian, seasonal, health, sleep, light, desynchrony

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BACKGROUND

The essential physiological role of the pineal hormone melatonin is to provide information on photoperiod (day length) for the organization of seasonal physiology (1). It does not appear to have an essential function in the circadian system but has clear modulatory effects. These functions depend primarily upon G-protein linked membrane receptors MT1 and MT2 (2, 3). It may also be concerned with periodicities other than circadian and seasonal but as yet this is an emerging field (4–6). Its profile of secretion reflects the length of the scotoperiod (night). Even humans with ubiquitious artificial light, can show this change in suitable conditions (7). It was originally called a photo-neuroendocrine transducer molecule and subsequently, informally, a darkness hormone. Most if not all vertebrate photoperiodic species depend on this signal to time seasonal breeding (1), but see (8).

Melatonin is synthesized from tryptophan via 5-hydroxytryptophan and 5-hydroxytryptamine (serotonin). Then *N*-acetylation of serotonin by *N*-acetyl transferase (arylalkylamine N-acetyl

transferase, AA-NAT) to *N*-acetylserotonin (NAT) and *O*-methylation by acetylserotonin O-methyltransferase (ASMT), [previously known as hydroxyindole-*O*-methyltransferase (HIOMT)] to melatonin (*N*-acetyl-5-methoxytryptamine). A major increase (7–150 fold) in the activity of AA-NAT at night is usually rate limiting in melatonin production. The rhythm of production is endogenous, being generated by clock genes in the suprachiasmatic nuclei (SCN), the major central rhythm-generating system or "clock" in mammals (9, 10). The rhythm, as for the circadian system in general, is synchronized to 24 h primarily by the light-dark cycle acting via the retina and the retinohypothalamic projection to the SCN (9).

Light of suitable intensity and spectral composition can phase shift and entrain circadian rhythms. Light also suppresses melatonin production at night (11). The amount of light required for suppression varies from species to species, with time of night, with spectral composition and with previous light exposure. In humans,∼2,000 lux full spectrum light (domestic light is around 50 to 500 lux) is required for complete suppression at night. However, much lower intensities will partially suppress and shift the rhythm (12, 13). A non-image forming photoreceptor system of light sensitive retinal ganglion cells is implicated in these effects, with a pivotal role of the photopigment melanopsin, although in normal circumstances input from rods and cones is also used (14, 15). In humans maximum suppression and phase shifting for equal numbers of photons is given by blue light (460–480 nm).

In humans melatonin is metabolized, ∼70% to 6-sulphatoxy melatonin (aMT6s), primarily within the liver, by 6-hydroxylation, followed by sulfate conjugation (with some species variaions). A number of minor metabolites are also formed, including the glucuronide conjugate. N1-acetyl-N2-formyl-5-methoxykynuramine and N1-acetyl-5-methoxy-kynuramine, were initially reported as brain metabolites (1, 16) but have proved difficult to detect in plasma or urine except after administration of exogenous melatonin (17). Exogenous oral fast release or intravenous melatonin has a short metabolic half-life (20 to 60 min, depending on author and species), with a large hepatic first pass effect and a biphasic elimination pattern (18). Slow release/prolonged release/surge sustained preparations are of course designed to extend the time of high circulating melatonin [e.g., (19)]. It has low bioavailability in general although transmucosal administration increases bioavailability (20). A critical feature of exogenous melatonin with regard to its clinical uses is its very low toxicity and lack of addictive properties (21, 22).

Source of Endogenous Melatonin

The pineal gland is the source of the vast majority of circulating melatonin in mammals [e.g., (23–25)]. Its synthesis and presence has been described in a large number of other structures, but they do not appear to contribute significantly to blood levels in, for example, humans and rodents, except following specific manipulations of synthesis such as provision of excess precursor (26, 27). Pinealectomy leads to loss of the rhythm and usually undetectable amounts of circulating melatonin in mammals although with high sensitivity assays traces may be found. This is curious in view of reported non-pineal melatonin synthesis, sometimes in very large amounts, and the highly lipophilic/amphipathic nature of the molecule which penetrates all compartments rapidly (28, 29). Superior cervical ganglionectomy (denervation of the pineal) also abolishes the rhythm (30). Retinal melatonin is of major interest [e.g., (31, 32)] but beyond the scope of this text.

Non-pineal melatonin has been considered to act locally (29). Local effects have been invoked with regard to metabolism, immune function, gut function, inflammation, membrane fluidity, mitochondrial function, apoptosis (both stimulation and inhibition), free radical scavenging, direct anti-oxidant activity, influence on anti-oxidant enzymes, redox status, radioprotection, and others (33). Protective therapeutic effects are reported with regard to many various systems but notably neural, oncological and cardiovascular. Some of these effects are thought not to require receptor signaling, although melatonin receptors are now found widely distributed.

Very recently it has been demonstrated that in the mouse brain melatonin is exclusively synthesized in the mitochondrial matrix. It is released to the cytoplasm, thereby activating a mitochondrial MT_1 signal-transduction pathway which inhibits stress-mediated cytochrome *c* release and caspase activation: these are preludes to cell death and inflammation. This is a new mechanism whereby locally synthesized melatonin protects against neurodegeneration. It is referred to as automitocrine signaling (34). Another recent addition to our understanding is the observation that a gut bacterium, *Enterobacter aerogenes*, expresses an endogenous circadian clock that is responsive to signals from the host's circadian system, the hormone melatonin, and changes in temperature. This establishes a prospective link between melatonin as a peripheral circadian zeitgeber (time cue), and the gut (35).

Peripherally administered exogenous melatonin (sometimes in very high pharmacological doses) can presumably access the various structures involved in local effects even though the non-pineal endogenously synthesized melatonin does not apparently get out. The gut is reported to contain several 100 fold more melatonin than the pineal gland, but does not contribute to the circulating rhythm of melatonin. It is said to sustain the (very low) day time plasma levels (27). Although melatonin is present in some foodstuffs (36), in the authors experience it is hard to show an increase in plasma melatonin after a normal meal. This area has been extensively reviewed by others and will also feature in this volume.

In principle the established role of melatonin in rhythmic function is not necessarily incompatible with the use of high doses for 'protective' effects. Unless desensitization of the melatonin membrane receptors occurs as a result of continuous high circulating concentrations (37, 38) and compromises functions responsive to low levels of melatonin such as sleep and circadian phase.

Melatonin Physiology

Seasonal Rhythms

A truly distinct physiological role for melatonin was initially indicated by the fact that pinealectomy or ganglionectomy (which

abolished the rhythm of circulating melatonin) abolished the ability of photoperiodic mammals to time seasonal physiology according to the day length (with very rare exceptions) (39, 40). Melatonin secretion, long in long nights, short in short nights provided the information, via photoneurotransduction, to body physiology for the organization and timing of seasonally rhythmic functions such as reproduction and coat growth. Replacement of the endogenous melatonin signal by long or short profiles of exogenous melatonin at the same plasma concentration as the endogenous signal was equipotent with day length for control of seasonal timing. The downstream events have now been investigated in considerable detail [e.g., (41, 42)] and melatonin treatment to shift the timing of seasonal breeding in domestic species such as sheep, mink, and goats to maximize profit is now commercialized (43).

Humans have residual seasonality as evidenced by numerous physiological variables. and particularly by the existence of seasonal affective disorder and its treatment by suitable light exposure (and on occasion by melatonin as a chronobiotic) (44, 45). So for the therapeutic use of melatonin in humans it should never be forgotten that this hormone has profound effects on animal seasonal functions. The evidence for anti-gonadotrophic effects of high amounts in humans is quite substantial (46–49), and an influence on pubertal development is possible but not demonstrated. For example photoperiod, via melatonin profile, times puberty in sheep (50), melatonin inhibits LHRH stimulation of LH in the neonatal rat pituitary (46, 51), and in a case report of successful treatment of delayed puberty in a young woman, her production of melatonin declined dramatically (52). A serious attempt to develop it as a contraceptive was made some 25 years ago (53).

Circadian Rhythms

By contrast it is quite difficult to show major effects of pinealectomy on circadian rhythms and even on sleep. Initial investigations on pinealectomized rats showed little effect on activity rest cycles (54). These have been reinforced by a very recent study, again indicating that removal of the pineal has no effect on rodent sleep (55). However, if animals were subjected to an abrupt phase shift (as in jet lag), they adapted faster to the new schedule without a pineal gland (56, 57). This suggested that the pineal, and by inference melatonin, acted as a brake on abrupt changes of phase, these being undesirable in a natural environment. This possibility is reinforced by the fact that suppression of melatonin production by the beta blocker atenolol leads to faster adaptation to light-induced phase-shifts in humans (58). Ironically therefore whilst exogenous melatonin is used to hasten adaptation, it is possible that a function of endogenous production is to do the opposite.

Further support for a modulatory role in the circadian system is evident from the fact that in constant bright light pinealectomized animals, in comparison to sham operated animals, show more disrupted rhythms in wheel running, general activity, body temperature, and heart rate (54). Several authors have suggested that a function of the pineal and melatonin is to act as a coupling agent with regard to rhythmic systems ("circadian glue"). This would fit with the suggested role of maintaining the status quo (**Figure 1**). It could also be considered with respect to any effects on other periodicities.

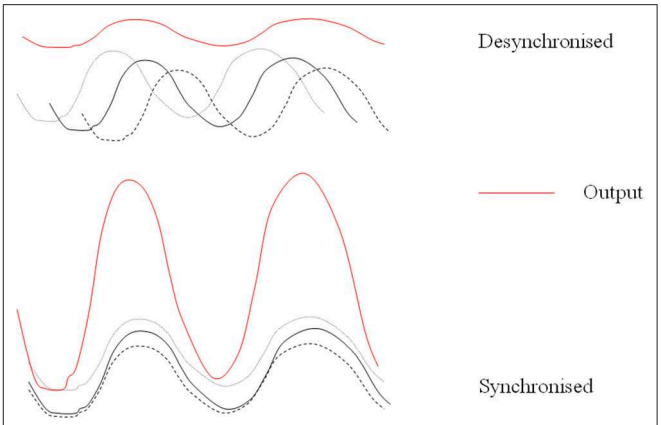

FIGURE 1 | Desynchronized rhythms lead to lowered output of a multi-oscillatory system. Simplified diagram of how melatonin might act endogenously to maintain coupling and synchronization of its target outputs and how desynchronized rhythms may lead to lowered production of melatonin itself.

Since melatonin is constantly referred to as the sleep hormone in the media, it is worth stating that it is not essential to sleep although we sleep better when in phase with rhythmic circulating melatonin and the rest of the circadian system (59). It is, to say the least, difficult to study pinealectomized humans before and after pinealectomy. However, this has been done prospectively with pre and post-operative polysomnography, the so-called gold standard for sleep measures. No effects of the missing pineal on sleep were seen (60). This was a small but careful and exceedingly rare study, it merits serious attention. The final comment on melatonin as a "sleep hormone" is that it most certainly is not so in nocturnal rodents—it is a darkness hormone not a sleep hormone.

COUNTERING CHANGES IN TIME CUES

The accumulated knowledge on the deleterious effects of abruptly changing time cues in for example shift work and jet lag [e.g., (61–67)] lead to the suggestion that one function of endogenous melatonin is to protect against abrupt short term changes of phase by maintenance of the circadian status quo.

Effects on Circadian Rhythms

Early work indicated that timed exogenous melatonin treatment, pharmacological in rats, close to, but still usually supra-physiological in humans, could entrain activity rest cycles in rats, shift circadian phase, assessed using endogenous melatonin as a marker rhythm, and synchronize free-running rhythms in humans. For references see (68).The most obvious manifestation in humans is the timing of the sleep-wake cycle. Phase shifts and entrainment after timed low dose melatonin treatment were evident initially in the rhythm of sleep and of melatonin itself and then in the timing of all the circadian rhythms observed

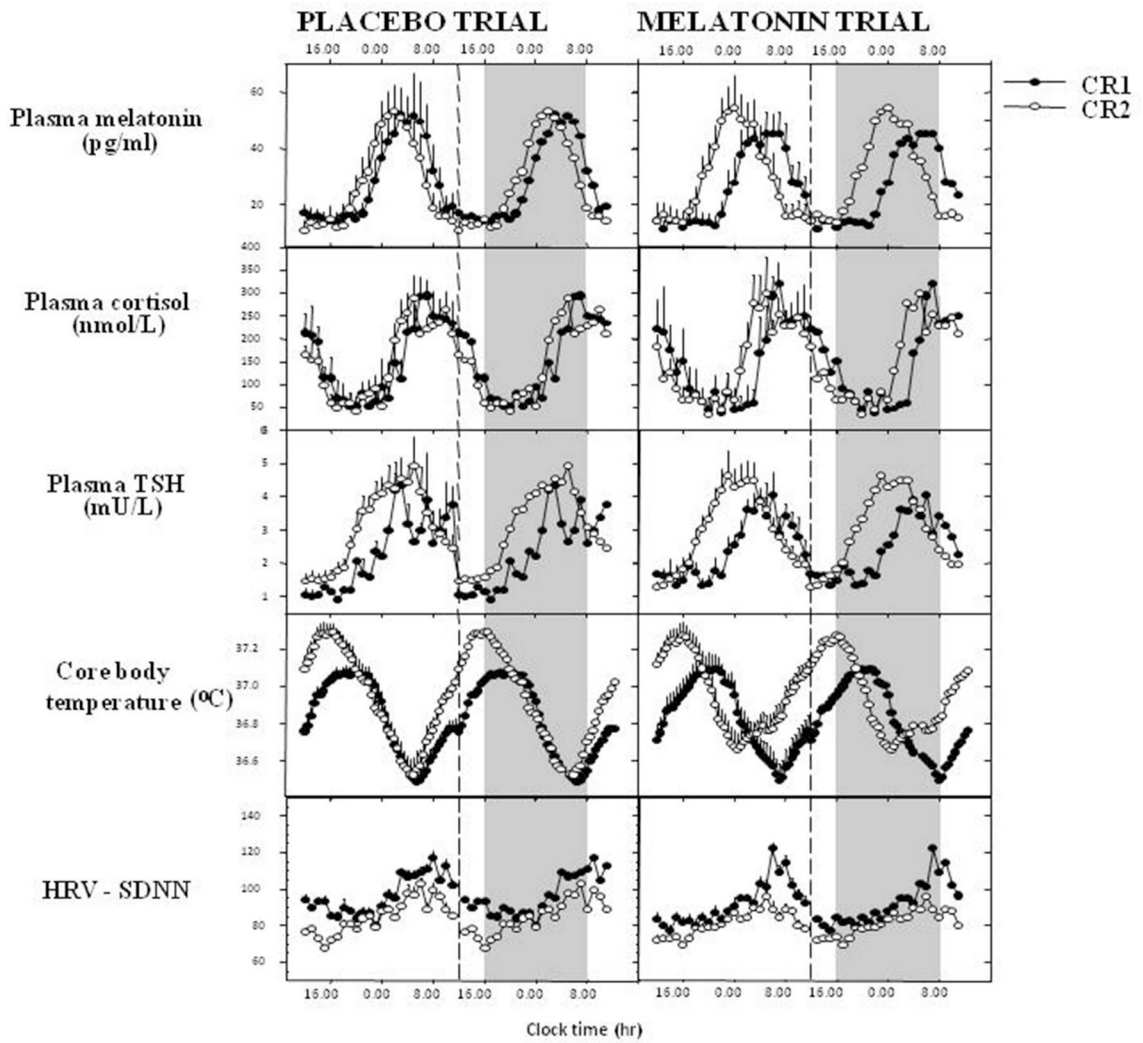

FIGURE 2 | Melatonin phase shifts all measured rhythms in humans. 1.5 mg surge sustained release at 1600 h daily for 8 days, recumbent, <5 lux, 1600–0800 h, evaluated in constant routine. Data derived and redrawn from Rajaratnam et al. (19), Middleton et al. (69), and Vandewalle et al. (71). CR1, 1st constant routine; CR2, 2nd constant routine; TSH, thyroid-stimulating hormone; HRV-SDNN, heart rate variability- standard deviation of the interbeat interval of normal sinus beats.

(19, 69, 70) (**Figure 2**). Both phase advances and phase delays can be induced dependent on the time of treatment (72) which can be expressed as a Phase Response Curve or PRC. The melatonin PRC is approximately the opposite of that to light pulses and for maximum effect the two treatments can be combined with careful timing (73, 74). If the period of melatonin secretion is considered to be "biological night" then low dose (0.5–5 mg) treatment in the late afternoon will advance circadian phase and sleep (75), whereas treatment in the early 'biological morning' will cause phase delays. Thus, it is important to know or predict circadian phase in order to time treatment correctly. The clearest demonstration of entrainment could be seen in free-running blind subjects (76, 77) and in sighted subjects kept in a time free environment (78).

Melatonin clearly has both a direct sleep inducing effect coupled with a circadian phase shift (70). Importantly it was shown to act directly at the level of the suprachiasmatic nucleus (79) to modify its rhythmic activity, amplitude and phase, via G-protein linked membrane receptors now extensively characterized as MT1 and MT2 (MTR1A/Mel1a, Mel1b/MTR1B) (2, 3, 79–81). MT1 was associated with suppression of SCN electrical activity and MT2 with phase shifts, with some redundancy and cooperation between these subtypes. However, a recent report describes a lack of overlap in mouse brain structures showing one or other of these receptors. The authors state that "the expression and distribution of MT2 receptors are much more widespread than previously thought, and there is virtually no correspondence between MT1 and MT2 cellular expression" (82). A third receptor MT1c is not found in mammals but a related G-protein coupled receptor GPR50 has 45% identity in amino-acid sequence with MT1 and MT2 and is thought to be the ortholog of Mel1c in mammals (83). It may have a role in

glucocorticoid receptor signaling with implications for peripheral control of circadian rhythms (84). Melatonin may also directly affect clock genes in the SCN [e.g., (85)].

Others will report in this volume in detail concerning receptors and downstream signaling events. It is just noted here that a recently reported signaling by melatonin receptors in the SCN appears to require G-protein-coupled inwardly rectifying potassium (GIRK) channels: a widely distributed physiological neural communication system (86). The authors propose this as some of the explanation for the variety of reported effects of this hormone in mood and other neurological disorders.

In addition to effects on the central rhythm generating system, melatonin also influences peripheral oscillators for example in the pars tuberalis, the cardiovascular system, the skin, the adrenal (87–90), various primate fetal tissues (91) and possibly the expression of sirtuin 1 (a histone deacetylase) which is thought to enhance circadian amplitudes and may prolong survival (92). Melatonin clearly has the potential to influence all rhythmic function by virtue of its universal distribution, however very recent data indicates a major role for glucocorticoids in entraining/synchronizing peripheral clocks (84). It is clear that melatonin can manipulate the circadian system. It may well be that a combination of melatonin, light and glucocorticoids could provide the most efficient realignment of both centrally generated and peripheral clocks.

Circadian Desynchrony

In a natural environment, changes in circadian rhythms occur due to seasonal influences, notably changes in photoperiod leading to shorter or longer melatonin secretion profiles (93). In humans a seasonal effect is more commonly seen as delayed rhythms in winter (94) especially in polar regions with no sunlight for long periods of the year (95). A duration change in melatonin profiles is rarely seen in humans in temperate zones but can be elicited with artificial light/darkness (7). one explanation is that the onset of secretion is delayed in winter, but the subject is required to get up to work in the morning. Thus, the full expression of the profile is curtailed by artificial light suppression both in the evening and in the morning. In the urban environment of today artificial light is everywhere leading to changes in sleep. It is interesting to compare sleep in similar rural communities with and without artificial light. Artificial light clearly impacts the timing and duration of sleep (96).

The abrupt changes in the light dark cycle and consequent desynchrony experienced by shift workers and time zone travelers are now known to be associated with increased risk of accidents, sleep deficits, lowered alertness and performance, gut problems, lowered fertility, perhaps psychiatric problems, and increased risk of major disease such as cancer, diabetes, metabolic syndrome and heart disease (67, 97).

The central pacemaker of the SCN adapts slowly to these abrupt changes and peripheral oscillators adapt at different rates, such that the body is in a state of both internal and external desynchrony (63, 65, 98–103). Melatonin (and other rhythms being driven by the SCN) is slow to adapt, endeavoring to maintain the circadian status quo. It is out of phase during adaptation and may be partly suppressed by sufficient light at night (LAN), e.g., in shift workers, although not all studies concur. This is thought to be a causal factor in the increased risk of, for example, breast cancer by some authors and will be discussed later (97, 104, 105). However, the entire circadian system is disturbed in these circumstances, not just melatonin.

Out of phase rhythms with or without suppressed melatonin may well be involved in the deleterious effects of shift work (63, 104, 106, 107). As yet there is no definitive linkage between a particular degree of melatonin suppression and any deleterious effects. Some people appear to lead perfectly normal lives with very low or even undetectable melatonin. But there is no long term information on disease risk in these low melatonin secretors. Certainly desynchrony will be one cause of disordered sleep, since we sleep better when in an appropriate phase relationship with the melatonin rhythm. But when the entire circadian system is dysregulated numerous other effects and potential causes can be invoked.

SLEEP

Melatonin itself and its agonists have been developed primarily to treat sleep disturbance (108) but with currently expanding possibilities for clinical therapeutics. Its immediate effects on sleep were initially investigated long ago, first by Aaron Lerner who identified melatonin. Its immediate effects on sleepiness/sleep are accompanied by a dose-dependent lowering of core body temperature in near physiological doses (75, 109), and this has been invoked as part of the mechanism of action. This phenomenon has been linked to sleep induction in a series of careful trials (110, 111). Melatonin has clear, time-dependent direct (soporific) and phase shifting effects on human sleep in near physiological/low pharmacological doses (70, 112) (see **Figure 3**). It's rhythm is closely associated with the timing of sleep and sleep propensity, and inversely with that of core temperature (113).

When treatment with melatonin is related to the so-called circadian rhythm sleep disorders (CRSDs) it is a logical development exploiting both the direct and phase shifting properties (68). CRSDs include delayed sleep phase, advanced sleep phase, free-running sleep, and the sleep detriments of jet lag and shift work. Although the endogenous central pacemaker has a major role in timing sleep, humans exercise choice according to desire or necessity, as to when they try to sleep. This means that sleep rhythms are not a pure manifestation of the circadian system. True circadian phase shift and/or entrainment requires a demonstration that a marker rhythm such as melatonin, cortisol or core temperature is entrained. If treatment is timed to maximize the phase shifting and direct sleep inducing effects of melatonin it can be very successful, particularly with respect to mistimed sleep.

Delayed Sleep Phase Syndrome (DSPS)

Typically a subject reports inability to sleep before 2 to 6 a.m. When not required to maintain their schedule—i.e., weekends, holidays, etc.—they sleep without difficulty, and will awaken spontaneously after a sleep period of normal length. Severe cases of DSPS are relatively common in adults (114).The incidence

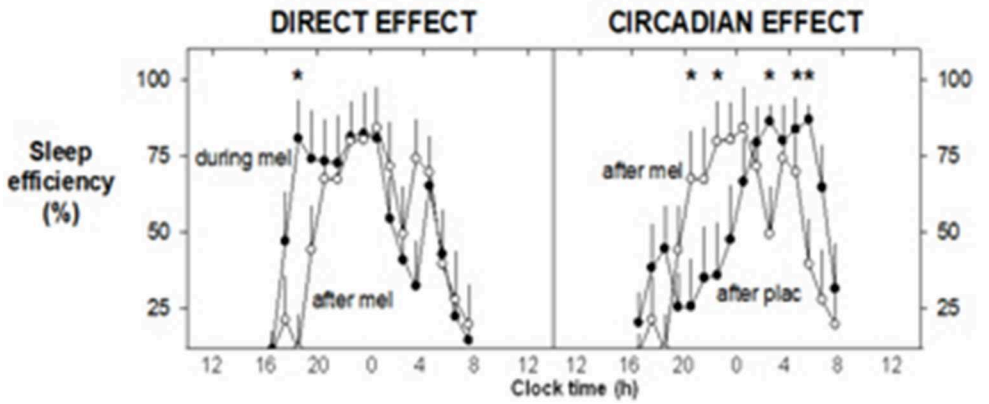

FIGURE 3 | Exogenous melatonin has both direct and indirect effects on sleep. 1.5 mg surge sustained release at 1600 h daily for 8 days, recumbent, < 5 lux, 1600–0800 h, evaluated in constant routine. Mean sleep efficiency levels (% per hour: $n = 8$). The direct, sleep-facilitating effect of melatonin **(left)** is illustrated by a comparison between sleep efficiency profiles on the last day of melatonin treatment and sleep efficiency on the following washout day. Increased sleep efficiency (direct effect) is observed for the first 2–3 h during melatonin treatment. The circadian effect of melatonin on sleep **(right)** is shown by comparing the sleep efficiency on the washout day (the day after melatonin or placebo). On the washout day, placebo was administered to all participants. A shift in the distribution of sleep can be observed after melatonin treatment, with the major bout of sleep occurring earlier in the sleep opportunity. On the corresponding day after placebo, the major bout of sleep occurred later in the sleep opportunity, although an initial rise in sleep efficiency is noted at around the commencement of the sleep opportunity. With Permission from Rajaratnam et al. (70). *Significant difference between CR1 and CR2.

of clinically important DSPS in students/adolescents may well be as high as 7%. An early meta-analysis (115) concluded that there was no evidence for efficiency of melatonin in treating secondary sleep disorders and sleep disorders associated with sleep restriction. Sadly they did not specifically select publications which gave treatment at the correct time but they did conclude that DSPS was an area where melatonin could be useful. The British Medical Journal published some "rapid responses" to this publication which were highly critical of the summary and conclusions.

Some very careful work has been carried out in adults and children with DSPS, measuring circadian phase and timing treatment to 5 h before melatonin onset for maximum phase advance. Sleep timing was advanced, criteria of general health were improved, there were no later effects on reproductive health and in some cases treatment could eventually be withdrawn. A meta-analysis (116) describes the quality studies in both children and adults published up to 2010 and concludes that melatonin treatment induced an earlier phase (melatonin onset, 1.18 h) reduced sleep latency of 23 min and earlier clock onset of sleep by 0.67 h. Timed melatonin treatment was recommended for DSPS by the American Academy of Sleep Medicine (117). More recent well-controlled trials have strongly supported the use of timed melatonin with or without timed light exposure for DSPS (118, 119). However, by no means all diagnosed DSPS patients have a circadian delay as well as a sleep delay (120).

For Advanced sleep phase syndrome (ASPS), there is little information on melatonin treatment.

Shift Work

Another common situation with temporarily displaced sleep is that of shift work. There is sparse evidence that melatonin can help day time sleep during real life night shift and night time sleep after return to day work, although anecdotally melatonin is used. An early real life study reported greater day sleep duration after night shift when subjects left work early (6 am, before conflicting bright light) and took melatonin (5 mg) before day sleep (121). A later real life timed study addressed both day and night time sleep and was successful in its carefully timed use of melatonin (3 mg) 1 h before bedtime (122). An increase in sleep duration of 15–20 min was obtained and a reduction in sleepiness at work (subjective measures). In a series of simulation shift work studies Eastman and colleagues have clearly shown that timed melatonin (1.8 mg sustained release) and bright light exposure will partially shift phase, such that day sleep is improved when working nights and subjects rapidly readapt on return to day work (73, 123, 124). Data from real life shift work studies were positive in one review (125) and the American Academy of Sleep Medicine approves its use in shift work sleep disorder (117).

Jet Lag

There are now so many reviews of the use of melatonin in jet lag, its dependence on timing and concomitant light exposure that it is pointless to write another here, see for example (98). In summary, successful studies used timed melatonin correctly, unsuccessful studies [e.g., (126)] did not. The latter in particular used a cohort who had flown from Norway to New York, stayed 4 days and then were studied on the flight back to Norway. One can predict that their study population was unadapted to New York time, phase shifted from Norwegian time, internally and externally desynchronized, and individually different since individual response to abrupt change of time cues is variable. Their lack of useful effect is not surprising since this situation was not taken into account.

In 2006 a Cochrane Database review concluded that melatonin was useful for jet lag (127). It is updated regularly. Timed melatonin treatment is recommended for jetlag by the American Academy of Sleep Medicine (128). Advice on how to time melatonin and light exposure can be found in reference (98) and elsewhere.

Sleep in the Elderly

Sleep problems in the elderly may be due to many factors one of which may be disturbed circadian rhythms. Prolonged release melatonin "circadin" is registered for use in sleep disorder of the over 55 s. From the European Medicines Agency Website: "Circadin was more effective than placebo at improving quality of sleep and the patients' ability to function normally on the following day. When the results of all three studies were looked at together, 32% of the patients taking Circadin (86 out of 265) reported a significant improvement in symptoms after 3 weeks, compared with 19% of those taking placebo (51 out of 272)." The CHMP decided that, although Circadin has only been shown to have a small effect in a relatively small number of patients, its benefits are greater than its risks," https://www.ema.europa.eu/en/medicines/human/EPAR/circadin.

Use of melatonin in the very elderly, particularly suffering from dementia, has been advocated but proved to have adverse effects in some studies (129).

Sleep in Children

There has been considerable interest in the pediatric use of melatonin for sleep disorder, initially in children with neurodevelopmental disorders, in spite of the possible adverse effects on reproductive function. A large multi-center RCT has been conducted in the UK- the MENDS study (130). Escalating doses from 0.5 to 12 mg 45 min before bedtime were used. The primary outcome was sleep duration by diaries, and objective measures (actigraphy) were also used. They were able to show (130, 131) 23 min longer sleep and shortened sleep latency by 45 min. Evidently this success has inspired further treatment. Pediatric use of melatonin for sleep problems has covered autism, ADHD and intellectual disability (ID) (132) and now has expanded hugely to more general use. According to a serious UK newspaper- The Guardian- there are safety concerns: Despite the fact it is not licensed for use by any other age group, (other than over 55 s) 117,085 people under 18 were given melatonin "off label"—the term used for when a drug is given for an unapproved indication or in an unapproved age group—to aid sleep in the 2017–18 financial year. (https://www.theguardian.com/society/2018/nov/02/rise-in-melatonin-use-to-help-children-sleep-leads-to-safety-warning).

Non 24 h Sleep Wake Cycle (Irregular Sleep Wake Cycle, Free-Running Sleep-Wake or Non-24)

This condition is the least numerous of the CRSDs but the most interesting. It is the expression of an individual circadian clock periodicity. Each person has their own periodicity usually slightly longer than 24 h (hence the tendency to delay over the weekend) which manifests itself in the absence of strong time cues, primarily the light dark cycle. Many blind people with no conscious or unconscious light perception cannot properly synchronize to the 24 h day (133–136). In expressing their own periodicity they drift away from normal clock time such that intermittently they will be in "night" phase during the day (e.g., secretion of melatonin, low alertness and performance) and day phase during the night (low melatonin, poor sleep). It has been described as a lifetime's intermittent jet lag.

Melatonin (usually 0.5–5 mg daily, lower doses than 3 mg are often better) is able to synchronize this non-24 h sleep wake cycle to 24 h in the vast majority of patients (76, 137) (**Figure 4**). Until a registered melatonin preparation became available in the UK (circadin) (138), patients had to obtain melatonin on a named patient basis. It is prescribed by Moorfields Eye Hospital (premier eye hospital in the UK). Unfortunately the completely blind do not often appear before a specialist, the condition is not correctly diagnosed, and is often treated with straight hypnotics (which do not work). A survey in New Zealand reported very little use of melatonin to correct this cyclic sleep disorder (139).

In 1986 a blind man rang me up, said he had non 24 h sleep wake disorder, and could he have some melatonin? He had seen my jet lag studies and worked it out for himself. After a very successful double blind placebo controlled cross over study [5 mg melatonin, (134)] he took this dose, prescribed on a named patient basis by his GP, for the rest of his life. He died 8 years ago, of prostate cancer at 83 years old, after 24 years use, refusing to lower the dose. We checked his biochemistry and hematology after 10 years treatment and all was normal for age. Since then quite a number of similar studies have found the same synchronizing effect on sleep, and from the year 2000 synchronization of the underlying circadian pacemaker was shown in most, but not all patients. The tendency is to start with very low doses and if necessary increase (or in one

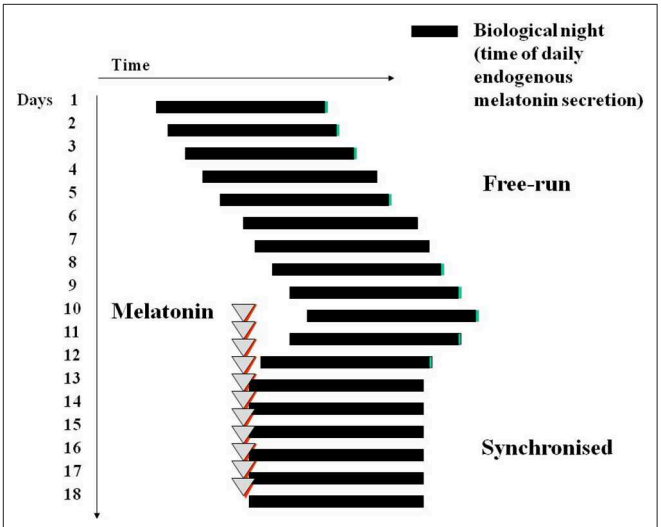

FIGURE 4 | Diagram of melatonin-induced entrainment by phase advance of a free-running sleep wake cycle and circadian phase, for example in a blind subject with no conscious or unconscious light perception. Treatment is best initiated in a period of good sleep prior to desired sleep time in the "biological dusk" before onset of melatonin secretion.

publication decrease) the dose until it is effective. We recommend starting treatment about an hour before bedtime when in a 'good' sleep phase. Even without full circadian entrainment sleep can be improved.

Non-specific Insomnia

As a treatment for non-specified insomnia melatonin also appears to be quite useful. Some 3,000,000 Americans used melatonin last year according to the following website (https://nccih.nih.gov/research/statistics/NHIS/2012/natural-products/melatonin), presumably for jet lag or for "poor sleep." In the case of non-specific "poor sleep" this is likely related in many cases to the fact that our circadian rhythms are frequently not in optimal phase in a "urban normal environment" (140) with insufficient time cues or zeitgebers to maintain optimum circadian phase. In these circumstances most people will delay the circadian system, particularly over the weekend if there is no requirement to get up in the morning. In this way the social need for sleep is in advance of the circadian optimum time and melatonin secretion in particular, and sleep suffers. The discrepancy has been referred to as "social" jet lag (141). Popping a melatonin pill in the evening has a good chance of advancing circadian phase to a more appropriate time and thus better sleep.

Melatonin and Cancer

Animal experiments have shown clearly the increased risk of cancer with abrupt phase shifts (97, 142, 143). In 2006 the World Health Organization in Lyon, France held a week-long meeting in which, on the basis largely of animal experiments, decided that shift work was a probable carcinogen (97, 144). A large proportion of the population of developed countries (15–20%) works shifts and thus this is of major health interest.

An association of the pineal gland with anti-cancer activity has a very long history (145). Early work suggested that the gland contained oncostatic activity initially not specified as melatonin. Important evidence of the association included for example that pinealectomy of rats led to much shorter survival times from DMBA-induced cancer and secondly that exogenous melatonin could substantially increase survival times (146). In the 1970s melatonin treatment (very large doses 80–300 mg pd) to young women was proposed for avoidance (prophylactic) of breast cancer (48, 53), and development proceeded to clinical trials in combination with a progestagen. These trials were not successful. But the subject continued of interest when light at night, thought to suppress melatonin (at least partially) (147–149), was invoked as the reason for an excess of breast cancer in nurses, working rotating long term night shifts (104, 144, 150), and subsequently other shift workers.

There is good reason to consider melatonin firstly as a prophylactic, in the case of suppression by light during night work, secondly to hasten adaptation of the circadian system to abrupt phase shift when this is desirable. Thirdly it has been very extensively researched with regard to its anti-cancer activity in breast cancer and other neoplasms (151, 152). Some of the most convincing data linking physiological levels of melatonin with anti-cancer growth concerns human breast cancer-mouse xenograft studies. In a series of experiments Blask et al. could show the protective effects of exogenous and endogenous melatonin and the deleterious effects of extra light (153–156). The xenograft approach is being applied to other cancers.

Most epidemiology agrees that there is an increased risk of developing various cancers as a function of long term night shift work (97, 150). Melatonin has been used as adjuvant therapy in various cancers for nearly 20 years notably by Lissoni et al. in very advanced cancer [e.g., (157)] with positive effects but not usually significant results. With all the suggestive background, several clinical trials in different cancers using melatonin, usually as an adjunct to conventional treatment, have now been conducted (33)—but not enough. The results are quite positive in several domains- survival time, progression of the disease, reduced toxicity of treatment and in general well-being. An important question is to what extent the effects are due to rhythm optimization and/or improved sleep?

METABOLISM

A substantial early clinical literature exists concerning diurnal and ultradian rhythms in metabolic function [e.g., (158)]. With the application of constant routine technology it became possible to identify endogenously generated (i.e., circadian) rhythms from those derived from the external environment, meal times etc. (159–161). This has now been extended to metabolomics. For example simultaneous evaluation of many metabolites in constant routine has shown that of 132 circulating metabolites nearly half showed a 24 h rhythmicity (162). Following sleep deprivation it was clear that many metabolites desynchronized amongst themselves (163, 164). With sequencing of the human genome, this approach has now devolved to the level of genes (67). Melatonin has been invoked as a supplementary treatment for avoidance or reversal of metabolic syndrome but without substantial evidence of efficacy [e.g., (165)].

The Entero-Insular Axis and Diabetes

The importance of rhythms to the entero-insular axis was also evident early on, with variations in glucose tolerance and insulin sensitivity (166). The subject has been very recently reviewed (161). The circadian, SCN-driven nature of these rhythms is now well established alongside the "masking" effects of mealtimes, meal content and other external inputs (167, 168). Triacylglycerol (TAG) has a particularly marked circadian rhythm in constant routine (167). During both simulated and real shift work, standard meals taken at inappropriate times at night—biological night when melatonin secretion is high-lead to evidence of insulin resistance/glucose intolerance and higher TAG, both risk factors for heart disease (167, 169). This is therefore one possible mechanism underlying the epidemiological data showing higher risk of these major diseases.

Circadian re-adaptation in real shift workers resolves some metabolic risk factors (169) (and see Gibbs M, Hampton SH, Morgan L, Arendt J. Effect of shift schedule on offshore shift workers' circadian rhythms and health, 2004. http://www.hse.gov.uk/research/rrhtm/rr318.htm). So there is good reason to use the chronobiotic properties of melatonin (and timed light exposure) to manipulate circadian phase. It remains to be

determined to what extent central and peripheral oscillators remain in synchrony/coupled in these circumstances.

Melatonin clearly influences glucose concentrations-pinealectomy leads to increased glucose in nocturnal rats (170). In MT1 and MT2 receptor knockout mice the SCN-driven glucose rhythm is abolished independently of peripheral oscillators in muscle, adipose tissue and liver (171). In humans in one study, the decrease in glucose tolerance from morning to evening was mostly influenced by the endogenous circadian system compared to the sleep-wake cycle. However, in apparent contrast to pinealectomy effects in animals, melatonin administered during day time just prior to a glucose tolerance test in healthy adults clearly impaired glucose tolerance both in the morning and the evening (172, 173), an effect that was dependent on a common gain-of-function variant of the melatonin receptor gene MTNR1B152 (see below). Melatonin may also acutely decrease insulin secretion in cultured human islets (174). Thus, some controversy exists in the literature especially when comparing results in nocturnal rodents with diurnal humans with both beneficial and detrimental effects of melatonin reported. It is intriguing to note that the rare condition 'familial insulin resistance' or Rabden-Mendenhall syndrome is associated with pineal hyperplasia (175, 176).

In view of pre-existing associations of the pineal and melatonin with metabolic function the discovery of related MT1 and MT2 receptor variants aroused enormous interest. A common variant in MTNR1B—*MTNR1B* rs10830963 is associated with increased risk of type 2 diabetes, increased fasting plasma glucose levels and impaired early insulin secretion (177, 178). Moreover, late dinner, associated with elevated melatonin concentrations (as in night shift workers, above), impaired glucose tolerance in "gain of function" *MTNR1B* risk allele carriers but not in non-carriers. These data suggest that circulating melatonin is related to the development of Type 2 Diabetes, in a deleterious sense. Of course sleep restriction is also associated with impaired glucose tolerance, increased risk of metabolic syndrome and/or diabetes (179, 180). So that the usefulness of melatonin to address sleep problems may well increase risk of metabolic abnormalities. Some controversies have arisen and have been reviewed (181). The question is not solved.

CARDIOVASCULAR SYSTEM

Rhythmicity is a cardinal feature of the cardiovascular system, with demonstrable involvement of the SCN (182). Considerable attention has been directed at research into the disorders of rhythmic events and the timing of pharmacological interventions e.g., for elevated blood pressure (183). Timing of treatment clinically with anti-hypertensive drugs is accepted and current practice (184). Does melatonin influence the cardiovascular system? A recent review gives a positive report (185) with regard to several cardiovascular effects. In a controlled experiment melatonin was able to shift heart rate variability in company with the major circadian rhythms of cortisol, core body temperature and TSH (71). Evidently this corresponds to an effect on the central circadian clock.

There is certainly some good evidence that melatonin can lower blood pressure at night in patients with essential hypertension and/or metabolic syndrome (186, 187). Possibly the accompanying increased day night amplitude of systolic and diastolic rhythms was equally important and indicative of strengthened function of the SCN. The mechanism involved is not clear. The improved sleep reported in the subjects may well have contributed to the result.

Melatonin has probably had more exposure as a potential cardiovascular protective agent, with respect especially to myocardial ischemia/reperfusion injury. Numerous animal experiments suggest beneficial effects in a meta-analysis, with anti-oxidant effects, free-radical scavenging, anti-apoptosis and/or involvement of MT1 receptor suggested as mechanisms (188). However, a later meta-analysis and experimentation using melatonin in a combination with minocycline and magnesium sulfate did not show efficacy (189). Several clinical trials appear to be ongoing.

USE OF MELATONIN AS A CIRCADIAN "MARKER" RHYTHM, PROVIDING INFORMATION ON THE PHASE AND TIMING OF THE CIRCADIAN SYSTEM FOR BASIC RESEARCH, TIMED TREATMENTS

The rhythmic production of melatonin, normally high during the dark phase in all species studied to date, is linked directly via neural connections to the activity of the central circadian clock or pacemaker in the SCN (9). It was possible to show that the rate limiting synthetic enzyme pineal AA-NAT activity is closely related to the plasma melatonin profile in rats (190), and that the plasma profile is closely related to that of saliva in humans (191). Moreover, the urinary excretion of 6-sulphatoxymelatonin (aMT6s) the major metabolite in rats and humans reflects faithfully the profile of plasma melatonin in humans (192, 193). Thus, the measurable melatonin/aMT6s profiles in plasma, saliva or urine provide a 'window' on the clock. The melatonin rhythm has been extensively used to investigate the characteristics of human circadian rhythms. It is considered to be the best circadian marker rhythm, at least for the moment (**Figure 5**).

The characteristics of melatonin secretion in normal healthy volunteers have been studied for many years with increasing technological sophistication. They have been reviewed previously on numerous occasions. Similarly numerous publications describe abnormalities in melatonin secretion related to pathology. However, what is hardly ever considered is the general circadian status of patients studied. For example if a state of desynchrony exists, then an amplitude reduction in centrally driven and possibly peripheral circadian rhythms is likely (**Figure 1**) and low melatonin is not a specific symptom but a reflection of rhythm status. Another consideration is whether low (or high) melatonin amplitude is a cause or a consequence of the pathological state.

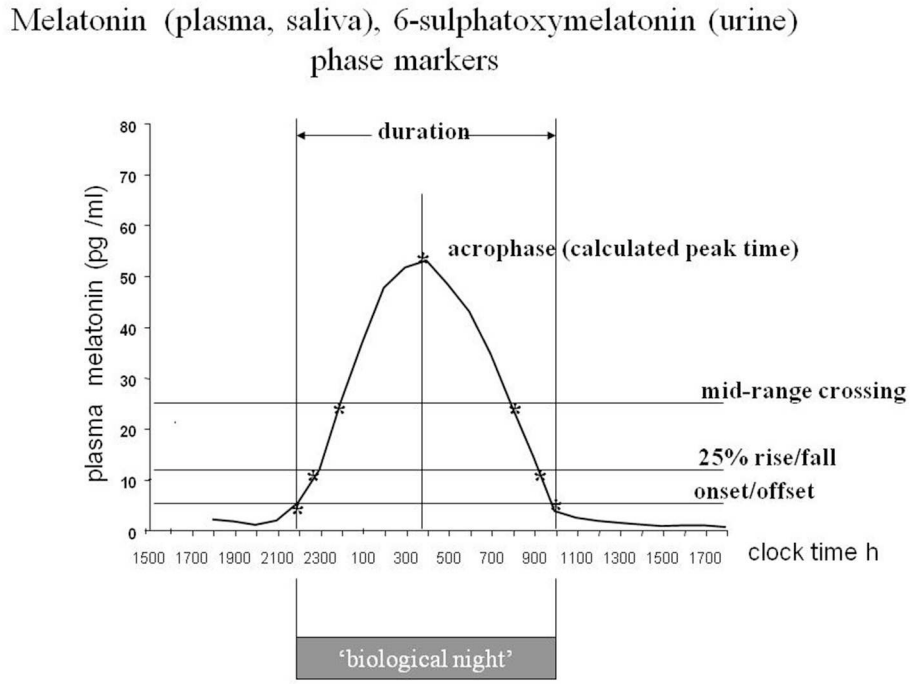

FIGURE 5 | The melatonin rhythm as a marker of circadian status. Diagram of a stylized plasma or saliva melatonin or urinary 6-sulphatoxymelatonin rhythm with the characteristics that have been used to define circadian status. Each body fluid has advantages and disadvantages from a practical point of view. Plasma is the most precise, with short interval sampling, saliva and aMT6s are the most useful for field studies. For long term monitoring of circadian status urinary aMT6s is well-tolerated. From Arendt (194), by permission.

A change in timing of the rhythm is easier to interpret, not least because there is normally such a vast difference in amplitude between individuals (195). This is probably the feature that has been most exploited clinically- but mostly for research purposes. The complete profile with sampling at hourly intervals or less provides the most information, but the timing of the onset of secretion in the evening in dim light, known as the DLMO (the dim light melatonin onset), is convenient and has been widely used to assess circadian status (196). First it should be noted that a large change in amplitude can look like a change in DLMO, depending on how the calculations are performed. The DLMOFF (dim light melatonin offset) in the morning is also useful as a circadian marker as is the "Synoff"—the time when production ceases (197). Urinary aMT6s provides less resolution, but even with 4 h day time/waketime samples and 8 h/oversleep the calculated acrophase is within 30 min of that derived from hourly sampling (193).

Each of the 3 matrices—plasma, saliva and urine, has advantages and disadvantages. Plasma is ideal and can be done overnight during sleep but requires catheterization and volume of blood loss is important. Saliva is practical but unless the subject is woken frequently for overnight sampling, the onset can easily be missed. Sequential urine samples have lower resolution but can readily be collected and measured by subjects in field studies, include the whole profile (much preferable to an early morning urine) and carried out long term.

For example workers on North Sea oil rigs collected, measured, aliquoted and froze urine continuously for 2–3 weeks whilst on the platform (198, 199). These samples provided a continuous record of circadian adaptation to night shift, or not depending on schedule. Similarly many blind subjects (135) and the crew of an Antarctic ship (200) have collected urine for 48 h at weekly intervals for 6 weeks or more. This approach provides an evolving picture of circadian status. It was particularly important in our work to judge the timing of melatonin treatment to entrain free running rhythms in blind subjects (76) and to find out to what extent particular shift schedules onshore, offshore and in Antarctica lead to desynchrony with associated sleep and metabolic problems (63). Melatonin profiling has been extensively used in research to provide a way of normalizing experimental subjects with diverse angles of entrainment relative to the sleep wake cycle, for comparative purposes.

WHY MEASURE MELATONIN?

In what clinical circumstances is there a need to know circadian status through melatonin measurement? Principally this is to identify desynchrony, delayed, advanced or free-running circadian status. Importantly it enables correct timing of treatment with melatonin and/or light or alternative zeitgebers as chronotherapies for disrupted rhythms, according to the appropriate PRC. Numerous drugs have large diurnal changes in pharmacokinetics which may or may not be circadian in nature

(184). The important question of timing of drug treatment has become more high profile in the clinic with the individualization of treatment regimes especially for cancer and the melatonin rhythm might well provide an individual circadian marker for timing specification (201).

THE UMBRELLA REVIEW

There are now so many reviews (systematic and narrative) and meta-analyses of the effects of melatonin on human and animal pathology that Posadzki et al. (33) have conducted what they refer to as "An umbrella review" or a "review of reviews" to pinpoint areas where a consensus may exist (**Box 1**). They identified 195 eligible articles according to their quality criteria, providing a valuable resource for evaluation of the evidence. Listed below and highly simplified are those effects and associations of melatonin in humans for which these authors found significant random effects in quantitative synthesis of eligible meta-analyses. The authors note that there is some overlap between the published meta-analyses which they identify. Furthermore, some of the analysis includes data from prolonged release melatonin circadin (two reviews) and melatonin agonists ramelteon (four reviews), agomelatine (two reviews), and tasimelteon (one review).

The conclusion from this tour de force is that the data supports the notion that endogenous and exogenous melatonin has benefits for health- a conclusion with which most people would agree. However, given the vast number of potential therapeutic uses for this hormone (as opposed to the accepted uses in sleep disorder) there are very few sufficiently large, randomized, multi-center, placebo-controlled, double blind trials in specific applications. Hopefully more are on the way.

Box 1 | Therapeutic effects and associations of melatonin, simplified, and condensed from Pozadski et al. (33).

In brackets: number of significant random effects/total number of analyses synthesized/total number of participants.

Risk of breast cancer up, related to low melatonin (3/3/3001)

Depression, response to treatment (1), remission (1), same study (2/6/1871)

Pre-operative anxiety down (1/2/761)

Post-operative anxiety down (1/1/73)

Post-operative pain down (1/1/524)

Prevention of agitation (1/1/170)

Safety high (1/1/2912)

Sleep latency down (3/4/6452)

Sleep quality up (2/2/5830), one study as for latency

Shown in a separate category are the significant random effects meta-analyses with insufficient data for quantitative synthesis:

In brackets: number of significant analyses/total number of subjects.

Breast cancer, risk of death at 1 year down (13 studies but no information on total number of subjects)

Nocturnal hypertension, systolic and diastolic, down (3/72)

Sleep latency down (5/2234)

Sleep duration up (4/2417), largest study significant for latency as above but non-significant for duration)

Melatonin onset (DLMO) (6/238)

Core temperature down (16/193)

A meta-analysis of the protective effects of melatonin in ischemic stroke in rodents is included with 432 animals and a highly significant large effect size.

CONCLUDING REMARKS

It seems that there are two major schools of research into the clinical therapeutic effects of melatonin. Firstly its association with biological rhythms from cells to organisms, and particularly with the timing and quality of sleep. It acts via well-characterized membrane receptors, which will be considered in depth by others in this volume.

Secondly in the last 25 years the expanding field of protective effects, often considered not to require receptor signaling, has come into being. From a philosophical point of view it would be very satisfying to reconcile these two approaches. It is probably true to say that "everything is rhythmic unless proved otherwise." If so, melatonin as a rhythm optimizer (synchronization, re-synchronization, entrainment, re-entrainment, coupling, phase and amplitude adjustment, phase and amplitude maintenance, periodicity), could well be invoked to explain many, even most therapeutic protective effects. Or at least a part of these effects.

It has been referred to as "circadian glue" but its influence on rhythms extends to other periodicities. Moreover, its "beneficial" effects on sleep may lead to a multitude of downstream events of therapeutic value. For example reducing the risk of insulin resistance, metabolic syndrome, obesity, diabetes, all of which have been associated with poor and/or insufficient sleep (see text). The association of melatonin with risk of cancer and therapeutic intervention therein, is strongly related to disordered rhythms.

Melatonin is good for human health, particularly via its ability to optimize sleep timing and often duration and quality with a multitude of downstream benefits. It can counter the debilitating effects of modern lifestyles: insufficient circadian time cues especially natural bright light, and exposure to artificial light at unsuitable times—the 24 h society. It is questionable whether or not long term use has deleterious effects and this is particularly important in pediatrics.

Circadian and other rhythm status, and reproductive function, during treatment with very large doses of melatonin needs investigation.

Finally, from personal anecdotal evidence, having taken melatonin in 2–5 mg oral fast release formulation on and off, mostly on, since 1981 after a mastectomy, I know that it does not prevent some of the pathologies associated with old age—osteoarthritis, Type II diabetes, spinal stenosis, uterine cancer. But I am still here!

DATA AVAILABILITY

All relevant data analyzed are included in this manuscript.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest Statement: JA is director of two companies Stockgrand Ltd and Surrey Assays Ltd which are concerned with measuring melatonin, 6-sulphatoxymelatonin, and other hormones. These companies had no influence on the writing of this text.

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REVIEW
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Melatonin Signaling a Key Regulator of Glucose Homeostasis and Energy Metabolism

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Melatonin, a hormone synthesized by both the pineal gland and retina, functions as an important modulator of a number of physiological functions. In addition to its rather well-established roles in the regulation of circadian rhythms, sleep, and reproduction, melatonin has also been identified as an important regulator of glucose metabolism. Recent genomic studies have also shown that disruption of melatonin receptors signaling may contribute to the pathogenesis of type 2 diabetes, although the exact mechanisms underlying its action remain unclear. Additionally, a large number of animal studies have highlighted a role for melatonin in the regulation of both glucose metabolism and energy balance. This review summarizes the current knowledge on the role that melatonin and its associated receptors play in the regulation of metabolism.

Keywords: melatonin, MT_1, MT_2, diabetes, leptin

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INTRODUCTION

Melatonin is a hormone predominantly secreted by the pineal of vertebrates. The synthesis of melatonin occurs during the night via a rather straightforward biosynthetic pathway that involves four different enzymes. In many vertebrates (e.g., fishes, amphibians, reptiles, and birds) pineal melatonin synthesis is directly controlled by the circadian clock located in the pinealocytes, whereas in the mammalian pineal gland the synthesis of melatonin is controlled by a circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Due to its lipophilic nature, melatonin is not stored within the pineal gland, but rather diffuses into the blood and cerebral spinal fluid where its levels in these fluids reflects its synthesis (1).

Melatonin acts on many different cell types within the body where it exerts its effects as an antioxidant and free radical scavenger or functions via its G-protein coupled receptors named melatonin receptor 1 (MT_1) and melatonin receptor 2 (MT_2) (2). MT_1 and MT_2 are expressed throughout the body where they regulate the entrainment of circadian rhythms, sleep, blood pressure, and reproductive functions. Since these effects of melatonin have been reviewed by other authors, in both this issue of the journal, as well as by recent reviews in other journals, we have decided to focus our review on the role that melatonin plays in the regulation metabolism. Additionally, in the last section of our review, we also summarize how modern genetic studies that have implicated melatonin receptor polymorphisms in the regulation of a number of different pathologies.

MELATONIN AS A REGULATOR OF METABOLISM AND BODY WEIGHT

Within recent decades, a large number of animal studies using both pinealectomized rats and melatonin receptor knock out (KO) mice have begun to establish a rather unexpected role for melatonin in the regulation of glucose metabolism and energy balance. Early pinealectomy studies demonstrated that abolishing melatonin levels produces glucose intolerance and insulin resistance (3, 4). Interestingly, reintroducing exogenous melatonin into this system restored metabolic parameters to levels observed within control animals. Similarly, in mice fed a high fat diet (HFD), exogenous melatonin administration was sufficient to restore diminished insulin sensitivity and glucose tolerance (5). Consequently, another study demonstrated that daily melatonin administration was sufficient to decrease the bodyweight gain of HFD fed rats by 54% compared to HFD rats not treated with melatonin (6).

These data suggest that melatonin may be functioning, at least in part, to alleviate a number of metabolic consequences associated with diet-induced obesity (DIO). To this end, we have recently examined the effects of DIO on body weight, food intake, and related metabolic parameters within WT and MT_1 KO mice fed a HFD. Our data demonstrate that DIO elicits markedly higher cumulative weight gain and hyperglycemia within MT_1 KO mice. Collectively, our data highlight that signaling through MT_1 may in fact regulate a number of protective metabolic responses during the course of DIO, thus raising the intriguing possibility that MT_1 may offer a unique therapeutic target for counteracting metabolic consequences elicited by DIO (**Figure 1**).

Previous studies have established a potential role for melatonin in the regulation of body fat by demonstrating that melatonin administration leads to a reduction in body fat content (7).

Moreover, correlations exist between aging and the reduction of pineal melatonin production, increased body weight, visceral fat, and high levels of leptin and insulin (7). In 1985, a seminal study by Bartness and Wade (8) demonstrated that in Siberian hamsters, melatonin treatment had the ability to decrease body weight, carcass lipid content, and food intake without affecting spontaneous locomotor activity. Years later, Rasmussen et al. (9) found that middle-aged rats treated with melatonin began to display decreased visceral fat, leptin, and insulin to levels that were found in young animals. In addition, in a crossover study, rats initially treated with melatonin rapidly began to increase body weight when switched to the control treatment, and rats that started as control animals rapidly decreased their body weight in response to melatonin treatment (10).

The progression of obesity is a complex process underlined by chronic inflammation (11). Interestingly, in a genetic mouse model of obesity, melatonin treatment was capable of ameliorating both inflammatory infiltration and obesity-induced adipokine alteration (12). Melatonin treatment has also been shown to reduce fat mass (and percentage of body fat) with a concomitant increase of lean mass in postmenopausal women following 1 year of treatment compared to placebo (13). Studies now suggest that melatonin treatment may stimulate lipolysis through the activation of the sympathetic nervous system, as well as stimulate intramuscular adipocyte lipolysis via activation of both extracellular signal-regulated kinase (ERK) 1/2 and protein kinase A (PKA) signaling (14, 15). Taken together, these studies begin to demonstrate a potential role for melatonin in modulating body weight, specifically through its regulation of fat mass.

To date, few studies have provided insight as to whether melatonin's regulation of body composition involves the modulation of feeding behavior. A recent study highlighted that MT_1 signaling stimulates the transcription *Pro-opiomelanocortin* (*Pomc*) mRNA in the hypothalamus and pituitary and the removal of this receptor results in mice spending substantially more time feeding (16). In addition, direct melatonin infusion via intracerebroventricular cannulation has been shown to reduce food intake (17), thus providing further evidence that melatonin might play an important role in modulating the circuitry that regulates feeding behavior.

INTERPLAY BETWEEN MELATONIN AND LEPTIN

Leptin is an adipose-derived hormone that is released in a circadian manner by adipose tissue. Plasma leptin levels peak late within the dark cycle and subsequently decrease during the light cycle (18, 19). Because of the circadian nature of melatonin's secretion—peaking at night—melatonin has been thought to be a circadian timing cue for leptin secretion. Indeed, melatonin has been shown to drive the daily rhythm of plasma leptin, and when it is absent the leptin rhythm is severely blunted in Syrian hamsters (20). Moreover, Buonfiglio et al. (17) demonstrated that the long-term absence of circulating melatonin leads to impairments in leptin signaling and leptin resistance within the hypothalamus. Obese individuals have high levels of leptin, however due to leptin resistance their regulation of food intake—and consequently body weight regulation—is impaired. Therefore, leptin resistance, which is induced either by pinealectomy or genetic removal of melatonin receptors, increases the expression of a number of orexigenic genes such as *Agouti-related protein* (*Agrp*) and *Neuropeptide Y* (*Npy*), which are modulated by leptin signaling.

These changes result in increased long-term food intake and weight gain. Interestingly, administration of exogenous melatonin prevented the negative effects induced by pinealectomy and reduced the expression of both *Agrp* and *Orexin,* thus leading to reductions in food intake, weight gain, leptin levels, and adipose fat pads (17). Consistent with these findings, Río-Lugos et al. (21) also demonstrated that melatonin treatment was able to decrease high levels of circulating leptin and adiponectin, as well as down-regulate the expression of *Npy*—a strong orexigenic signal—that was observed in HFD fed rats.

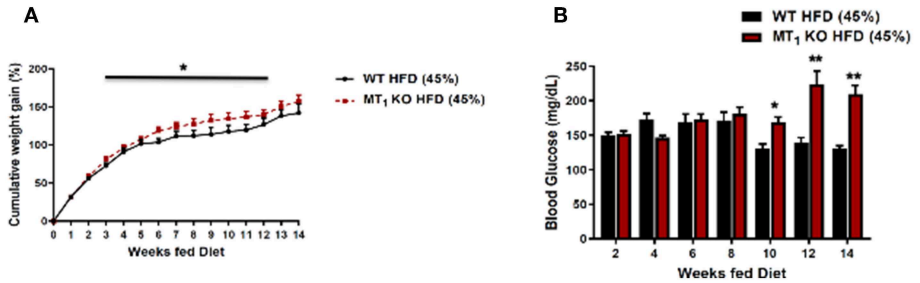

FIGURE 1 | Removal of MT_1 alters the metabolic response of C3H mice to DIO. Male mice were fed *ad libitum* with a HFD (D12451, 45% kcal/fat, Research Diets Inc.) from weaning at 4 weeks of age until 20 weeks of age. **(A)** MT_1 KO mice showed a small, but significant cumulative weight gain with respect to control **(B)**. Fasting glucose levels were significantly higher in MT_1 KO mice after 10 weeks of HFD. Data are presented as mean ± SEM ($n = 8$–10); $^*p < 0.05$, $^{**}p < 0.01$ WT vs. MT_1 KO Two-Way ANOVA (*post-hoc*: Holm-Sidak).

Finally, a recent study reported that MT_1 KO mice are leptin resistant compared to controls since the administration of leptin failed to induce signal transducers and activators of transcription 3 (STAT3) phosphorylation in the arcuate nucleus (22). Consistent with these results, leptin receptor mRNA levels in the hypothalamus of MT_1 KO were reduced (about 50%) with respect to mRNA levels in controls (22). Thus, the lack of MT_1 signaling induces leptin resistance by down-regulation of the leptin receptor.

MELATONIN AND ENERGY EXPENDITURE

In addition to the modulatory role of melatonin on energy intake, it seems that melatonin may also play an important role in modulating energy expenditure. In an experimental model of obesity and type 2 diabetes mellitus, using Zücker diabetic fatty rats, melatonin treatment induced browning of inguinal white adipose tissue (WAT) and increased Brown adipose tissue (BAT) weight with thermogenic properties (23, 24). Buonfiglio et al. (17) has demonstrated that pinealectomy decreased the amount of uncoupling protein 1 (UCP1) in BAT, thereby indicating lower thermogenic activity after cold exposure (25). Tan et al. (26) suggested that there is a correlation between light exposure at night and bodyweight gain in humans, and if melatonin recruits BAT in humans—as it does in other species—individuals who have their endogenous melatonin decreased by experiencing long daily photoperiods should have less functional BAT and may gain more bodyweight.

Recent studies suggest that gut microbiota composition is also correlated with metabolic disorders and obesity (27, 28). Surprisingly, it was demonstrated that melatonin treatment following DIO in mice, was capable of modulating gut microbiota back to levels observed in lean mice, and provides beneficial effects against obesity, insulin resistance, liver steatosis, and low-grade inflammation in HFD-fed mice (29). Although there remains much that still needs to be understood regarding the role of melatonin in energy homeostasis, there is significant data in different species that now demonstrates that melatonin has an anti-obesogenic effect and that it is involved in the regulation of all three of the main steps of energy balance: energy intake, energy storage, and energy expenditure.

MELATONIN RECEPTORS INVOLVEMENT IN THE REGULATION OF GLUCOSE METABOLISM

Collectively, much of the work obtained using mice lacking melatonin receptors has established a generally beneficial role for melatonin on glucose metabolism. Recent studies using these models have demonstrated that in the mouse genetic ablation of MT_1 or MT_2 affects glucose metabolism. MT_1 KO mice display systemic insulin resistance marked by impaired skeletal muscle glucose uptake, adipose tissue glucose uptake, and significantly reduced liver insulin sensitivity (30). This effect appears to involve modulation of phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT) activity and fits nicely with previous work demonstrating the ability of melatonin to inhibit hepatic gluconeogenesis (31), and stimulate glucose uptake within both skeletal muscle cells (32) and primary adipocytes (33). MT_2 KO mice, which previously lacked a clear metabolic phenotype, were recently reported to display decreased hepatic insulin sensitivity and increased insulin secretion (34). These findings differ quite drastically from a previous report demonstrating that MT_2 KO male mice are neither insulin resistant nor glucose intolerant (35) (see also **Figure 2**). The discrepancy between these findings may lie in the fact that the earlier metabolic characterization of MT_2 KO mice utilized male mice (35) whereas the more recent study was performed utilizing female mice (35). Sex differences in insulin sensitivity have been reported for both humans and rodents (36, 37), and as such, the divergence in these findings raises the intriguing possibility that melatonin receptors may potentially be regulated differently between males and females. Additional studies need to be done to confirm that gender is in fact a variable responsible for the observed differences in insulin sensitivity within MT_2 KO mice.

Thus far, efforts in understanding the role of melatonin on glucose metabolism have been focused heavily on pancreatic islets (38). Although valuable, this focus somewhat overshadows

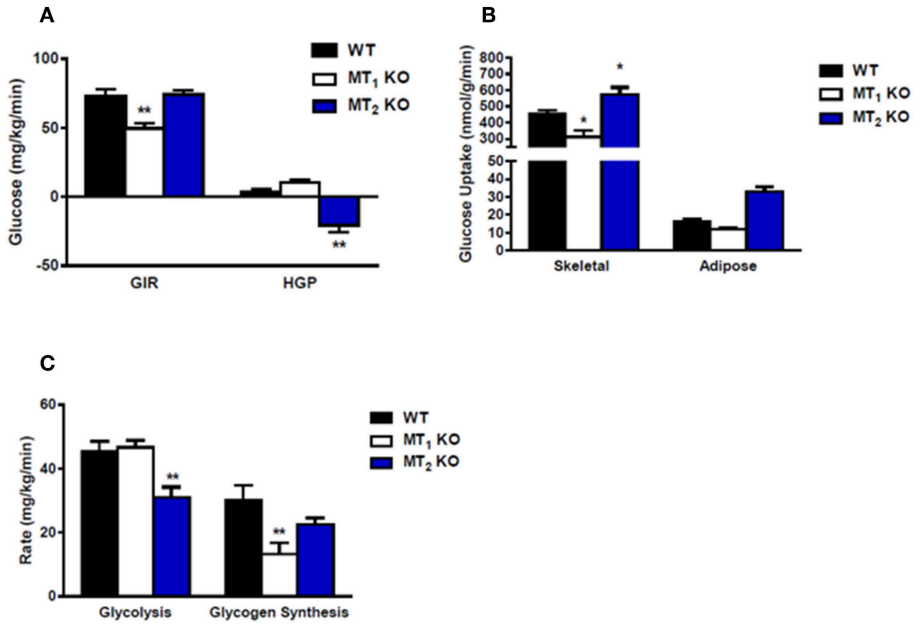

FIGURE 2 | Loss of MT_2 does not induce systemic insulin resistance in male mice. **(A)** Hyperinsulinemic-euglycemic clamp in awake mice. Glucose infusion rate (GIR) was not different between WT and MT_2 KO mice, whereas hepatic glucose production (HGP) was significantly lower in MT_2 KO mice. However, since negative HGP rates are not physiologically possible, the values observed in MT_2 KO mice likely arise from a slight underestimation of glucose disposal rates. **(B)** Insulin-stimulated glucose uptake in skeletal muscle (gastrocnemius) and white adipose tissue (epididymal) is higher in MT_2 KO with respect to WT and MT_1 KO. **(C)** Whole body glycolysis and glycogen synthesis. Glycolysis was lower in MT_2 KO mice while glycogen synthesis was not different between MT_2 KO and WT. Taken together these data suggest that while male MT_1KO mice show systemic insulin resistance with respect to WT, MT_2 KO do not exhibit insulin resistance. Results are expressed as mean ± SEM ($n = 5$–7 WT, MT_1 and $n = 12$ MT_2; Two-way ANOVA $^*P < 0.05$; $^{**}P < 0.01$, *post-hoc*: Holm-Sidak). Hyperinsulinemic-euglycemic clamp was performed as described in Owino et al. (30).

the effects of melatonin on other tissues (skeletal muscle, adipose tissue, liver), as well as its effects on the brain, where both melatonin receptor subtypes are highly expressed within the SCN (2). In the SCN, MT_1, and MT_2 function intricately together to entrain and synchronize the rhythm of the master circadian clock (39–41). It is now recognized that disruption of the circadian clock is linked to a number of metabolic disorders (42), and as such, the contribution of melatonin to clock entrainment and its potential implications on metabolism should not be overlooked in the design of future studies. The disruption of independent peripheral tissue clocks within insulin sensitive tissues has also been linked to a number of metabolic disorders (43–46). However, to date, current studies do not lend strong support to the notion that melatonin directly regulates metabolic parameters through its regulation of peripheral tissue clocks. Examination of the effect of melatonin receptor removal on clock genes within the pancreas, adipose tissue, and liver demonstrate only marginal effects on clock gene expression, amplitude and phase (47, 48).

Since the majority of the studies discussed above are performed in rodent models, another important point that must be considered in the interpretation of these results is that rodents are nocturnal and undergo a peak in melatonin synthesis during their active phase, whereas humans are diurnal and experience peak melatonin levels during their inactive "sleep phase." This difference suggests there may exist different functional requirements for both direct and indirect effects of melatonin between rodents and humans. Interestingly, a number of studies now highlight the importance of "timing" and the "delayed effects" of melatonin in interpreting results from melatonin signaling studies. In the case of insulin signaling, this was eloquently shown in a recent study highlighting the ability of nocturnal activation of MT_1 to modulate insulin sensitivity during the day (30). Moving forward in attempts to reconcile data collected from human and rodent models, it will be important to keep in mind potential differences that may exist pertaining to both the timing and functional constraints of melatonin in each species.

It is worth noting that a series of recent studies have reported that melatonin synthesis occurs in the mitochondria of neurons where MT_1 receptors are also present (49). Since mitochondria are key regulators of cellular metabolism, it would be interesting to see how dysfunction of melatonin synthesis and signaling within the mitochondria may contribute to the regulation of energy metabolism and expenditure.

ROLE OF MELATONIN RECEPTORS VARIANTS IN HUMAN METABOLIC DISORDERS

Over the years, genetic variants in melatonin receptors have been associated with a number of metabolic disorders such as type 2 diabetes (T2D), gestational diabetes mellitus (GDM) and

obesity (50–55). Recently, there has been considerable excitement directed toward the role of melatonin in the regulation of T2D. Much of this interest has come from a series of recent genomic-wide association studies (GWAS) linking *MTNR1B*, the genetic locus encoding MT_2, to increased fasting blood glucose levels and T2D risk (56, 57). An early study examined the association of six synonymous *MTNR1B* variants (G24E, L60R, V124I, R138C, R231H, and K243R) with obesity and T2D in a Danish and French population (57). None those variants were associated with T2D; however, G42E (rs8192552) was associated with decreased fasting plasma glucose. Furthermore, rs8192552 was also found to be associated with an increased prevalence of obesity, increased body mass index (BMI), and waist circumference.

In total, forty synonymous *MTNR1B* variants were later found to be associated with T2D. From these forty variants, four complete loss of function variants (A24P, L60R, P95L, and Y308S) – which lost the ability to bind melatonin and activate downstream Gi dependent signaling—were uncovered (58). In 2018, the same group under the lead of Karamitri reported their assessment of all forty variants on multiple pathways thought to be regulated by *MTNR1B* activation (58). They found that in addition to the four loss of function variants (A24P, L60R, P95L, and Y308S), four additional variants (S123R, R138C/H/L, F250V, and R316H) remarkably impaired cAMP responses (58).

A study from the DIAGRAM (DIAbetes Genetics Replication and Meta-analysis) consortium further aimed to identify a credible set of variants that overlap with FOXA2 binding sites as a causal mechanism for T2D susceptibility. Interestingly, *MTNR1B* variant rs10830963 was implicated as driving T2D association (59). A subsequent group did not manage to identify any association of rs10830963 and rs1387153 with T2D and fasting blood glucose (FBG) in their Indian population (60). However, when their data was subsequently stratified according to BMI, rs1387153 showed a strong association with low FBG levels in low BMI groups. Similar results were reported by Gan et al. in a Chinese population where rs10830963 didn't show an association with T2D (61). Although not associated with T2D, rs10830963 was found to be associated with GDM in a European cohort (62).

Collectively, genetic studies highlighting the association of *MTNR1B* to T2D risk have reconciled on the ability of two frequent variants (SNPs rs1387153 and rs10830963) to modulate insulin secretion from pancreatic beta cells (55, 57, 62–65). Interestingly, carriers of the rs10830963 risk allele express substantially higher levels of *MTNR1B* mRNA within their pancreatic islets when compared to non-carriers (57). This observation has fueled the general notion that increased melatonin signaling—as a consequence of increased receptor expression levels—likely inhibits pancreatic insulin secretion and increases T2D risk. These results suggest an overall *negative* effect of melatonin on diabetes progression; however, it is important to note that increased *MTNR1B* expression at the mRNA level may not correlate to increased receptor protein levels and/or receptor signaling within these islets. Moreover, these findings appear to be in conflict with a recent clinical study demonstrating that decreased nocturnal melatonin levels are in fact associated with increased, not decreased, risk for diabetes (66).

Although *MTNR1A* variants have not been directly associated with T2D, a few studies have reported an association of *MTNR1A* variants with polycystic ovary syndrome (PCOS). Patients affected by PCOS often develop insulin resistance and T2D. Finally, it is worth mentioning that a reduction in *MTNR1A* mRNAs has been observed in the liver of T2D patients that were unable to control glucose levels (30). Thus, experimental evidence also supports a possible role for MT_1 in T2D (30, 53, 67).

CONCLUSIONS AND FUTURE PERSPECTIVE

The experimental evidence accumulated thus far indicates that melatonin plays an important role in the regulation of glucose and metabolism are quite solid with a substantial amount of work conducted in human studies. Nonetheless, there continues to be a need for more conclusive studies to fully elucidate the exact role which melatonin and its associated receptors contribute to the regulation of different metabolic processes within the body.

AUTHOR CONTRIBUTIONS

SO, DB, CT, and GT wrote the paper. SO drew the figures.

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Single Cell Sequencing of the Pineal Gland: The Next Chapter

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The analysis of pineal cell biology has undergone remarkable development as techniques have become available which allow for sequencing of entire transcriptomes and, most recently, the sequencing of the transcriptome of individual cells. Identification of at least nine distinct cell types in the rat pineal gland has been made possible, allowing identification of the precise cells of origin and expression of transcripts for the first time. Here the history and current state of knowledge generated by these transcriptomic efforts is reviewed, with emphasis on the insights suggested by the findings.

Keywords: pineal, single cell sequencing, melatonin, transcriptomics, adrenergic, transcriptome profiling

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INTRODUCTION

The pineal gland is composed 90–95% of pinealocytes, which synthesize melatonin (1). Studies of the pineal gland have addressed the levels of transcripts involved in this process and have experienced remarkable improvements, innovations, and enhancements, in parallel with advances in cell biological techniques that have characterized the field. In general, genes expressed exclusively in non-pinealocytes have been ignored.

The first efforts to study a single mRNA transcript in the pineal gland came from northern blot analysis in the late 1980s (2–5). It required the equivalent of several rat pineal glands (4–5 mg wet weight); RNA was extracted, electrophoresed and blotted. This allowed for the radiochemical detection of transcripts encoding tryptophan hydroxylase1 (*Tph1*) and acetylserotonin methyltransferase (*Asmt)*/hydroxyindole-*O*-methyltransferase, the transcripts that encode the first and last enzymes in melatonin synthesis, respectively. The northern blot technique was highly useful, especially because it allowed the resolution of distinct molecular species.

However, it was obviously limited by the amount of tissue required and the small number of transcripts it could detect on repeated stripping and probing of blots.

The reverse transcription polymerase chain reaction was introduced into the pineal literature early in the 1990s (6–13). It was highly popular because it was sensitive and allowed multiple transcripts to be measured using small amounts of mRNA. It was used to detect low levels of transcripts including receptors and clock genes. However, quantitation with the method was somewhat unreliable and results could only reflect changes in small regions of mRNA amplified by the technique, which permits off-target results and precludes examination of the entire transcripts, which may have reflected gene leakage. Another problem with PCR was overamplification of very weakly expressed transcripts. In addition, analysis of each transcript was hands-on, limiting the number of transcripts that could be detected on a routine basis.

A revolutionary method was introduced to pineal cell biology with cDNA arrays, which at the start allowed for the detection of several hundred targets (14) and ultimately developed into microarrays, which permitted thousands of targets to be probed simultaneously using as little as one rat pineal gland (15–19) or 10 larval zebrafish pineal glands (20). However, this technique had the disadvantage of probing only portions of a transcript and was only useful for those transcripts which were represented on the microarray chip. Putting aside these limitations, this technique made important advances by reducing the amount of tissue required and increasing the number of genes probed. In the case of the rat pineal gland, it revealed large day/night changes in hundreds of transcripts, many more than had been realized at the time (19). The technique was also useful in comparing the pineal gland and retina and in determining the large number of genes shared by these two tissues.

The limitations of the cDNA chip technology were rapidly overcome in the early years of this century with the development of methods that sequenced the entire transcriptome, also referred to as bulk sequencing. Sequences have been obtained for chicken, human, mouse, rat, rhesus, and zebrafish pineal glands (21–30). This provided the sequence of full length transcripts, including the coding and flanking regions. It also provided an indication of splicing and alternative polyA sites. It sequenced all transcripts, known and unknown, including noncoding long and short RNAs (21, 29, 31). The technique is remarkably sensitive, allowing for tens of thousands of transcripts to be sequenced with the mRNA from a fraction of a single rat pineal gland.

As applied to the pineal gland, this technique provided excellent data on day/night differences. Moreover, studies on the rat pineal gland have provided valuable information on the effects of superior cervical ganglionectomy (SCGX) or decentralization (DCN) in *in vivo* experiments, and the effects of norepinephrine or dibutyryl cyclic AMP in *in vitro* experiments (**Figure 1**) (25). These confirmed and expanded previous results on the rat pineal gland, which showed that there was a broad change in the transcriptome on a 24-h basis. It also showed that neural stimulation of this tissue, in the form of postganglionic projections from the superior cervical ganglia stimulated the gland, based on the observation that both forms of surgical denervation, SCGX and DCN, blocked these changes. In addition, it revealed that most of these changes could be driven *in vitro* by norepinephrine or by its second messenger cyclic AMP. It is noteworthy that comparison of the transcripts that were induced more than 4-fold at night and by norepinephrine or dibutyryl cyclic AMP were nearly identical, numbering about 50 [Table S1 in (25)]. This correlation supported the view that the day/night differences were driven by a norepinephrine-cyclic AMP mechanism. It should be noted that the correlation was lower with weakly induced genes, which may be a reflection of statistical variation.

The development of advanced sequencing methods has evolved and deserves brief mention here. A hybrid approach is now available that combines Illumina short-read/high-throughput RNA-Seq with targeted qPCR and long-read Pacific Biosciences SMRT sequencing. In pineal gland studies it has been possible to identify 20 alternative RNA isoforms of the Ttc8/BBS8 gene (23). This gene was known to exist in multiple isoforms and is of interest because of evidence that it is involved in the Bardet-Biedl syndrome and non-syndromic retinitis pigmentosa (32–35). This technique is severely limited by the number of genes it can detect on a practical basis, but holds great promise for the study of isoforms, a complex and difficult endeavor. The interested reader is referred to the original publication for more details (23).

The most recent advance in sequencing is single cell RNA sequencing (scRNA-seq) (36). It takes several forms all of which allow for thousands of single cells to be sequenced simultaneously, yielding several thousand transcripts per cell. Overall, the technique has extremely high sensitivity and generates an enormous amount of data on the transcriptomes expressed in individual cells.

The technique was introduced into the pineal literature because of the suggestion that there were two populations of the cell that were defined by large differences in ASMT protein (37). As mentioned above, ASMT is the last enzyme in melatonin synthesis and converts the melatonin precursor N-acetylserotonin to melatonin. We hoped that the new technology would provide a transcriptional profile of each cell type and answer the question of whether pinealocyte subtypes defined by different levels of ASMT exist.

SINGLE CELL RNA SEQUENCING

Cell Isolation

The isolation of single pineal cells followed a well-established method, which has been used for biochemical, electrophysiological, and cytochemical studies (38–42). Glands were removed, soaked in DMEM solution and then cleaned under a microscope to limit the contaminating cells coming from blood and connective tissue. The glands were then placed in a freshly prepared Papain Dissociation System (Worthington; Lakewood, NJ) containing DNAase; details of the procedure have been published (43).

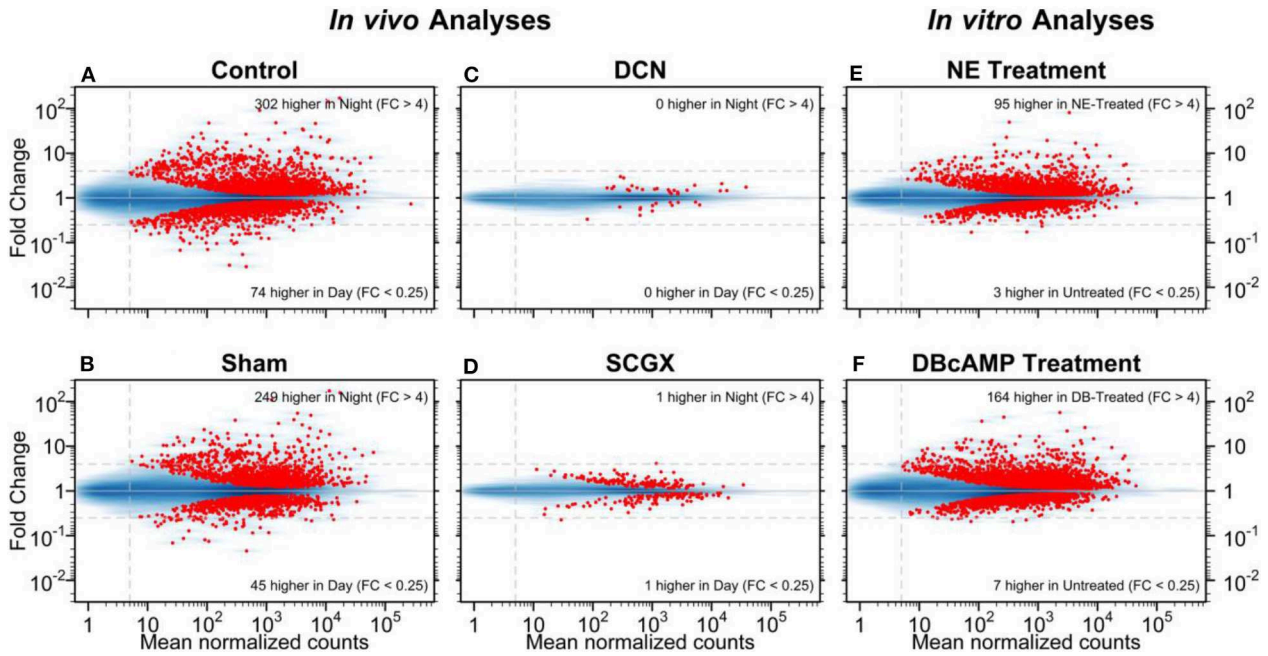

FIGURE 1 | Day/night differences in gene expression. The mean normalized read-pair counts (x-axis) vs. the estimated fold change (y-axis) are displayed on a log-log scale. Four *in vivo* and two *in vitro* analyses are presented as MA plots. The blue shading indicates the density of genes, and each red point represents a gene with statistically significant differential expression (adjusted-$p < 0.001$). Dashed horizontal lines mark 4-fold changes in both directions; the dashed vertical line indicates the minimum abundance threshold for the statistical tests. The four *in vivo* analyses compared night and day time points in adult rats for the following groups: **(A)** no surgery (Control); **(B)** neonatal sham surgery (Sham); **(C)** neonatal superior cervical ganglia decentralization (DCN); **(D)** neonatal superior cervical ganglionectomy (SCGX). The two *in vitro* analyses compared treated/untreated pineal glands: **(E)** norepinephrine-treated (NE) vs. untreated and **(F)** dibutyryl-cyclic-AMP-treated (DBcAMP) vs untreated. Reproduced from Hartley et al. (25). This figure and associated legend is published with permission of the original publisher under license CC0.1.0.

Single Cell Analysis

Single-cell cDNA libraries were constructed using a Chromium Controller (10X Genomics; Pleasanton, CA) and the Chromium Single Cell 3′ Reagent Kits v2 (43). In brief, dilute solutions of completely dissociated preparations of single cells were introduced into a stream of oil to make microdroplets. Sequencing reagents in the stream included a "sponge" that contains a unique cDNA marker for identification of the cell source of each transcript: one marker—one cell. This unique cDNA marker was incorporated into the mRNA at the polyA end, thereby providing a means of tracking the originating cell source of each molecule.

The microdroplets containing individual cells were mixed together and sequenced (Illumina HiSeq2500, Illumina; San Diego, CA). Ninety-eight bp sequences were produced in close proximity to the polyA tails. It was possible to recover 2,400–4,300 cells per sample, with 40–70 k reads per cell and 2,700–3,000 genes per cell detected on average (43).

The analysis of sequenced single-cell libraries was done by generating gene-level counts with the CellRanger analysis software v2.1.0 (10X Genomics). This aligns sequencing reads to the rat Rnor6.0 reference genome (Ensembl). The sequenced cells were subsequently filtered to remove doublets and low abundance genes. Dimensional reduction analysis was done (Seurat v2.2.0 package for R). Gene counts were normalized to 10^4 molecules per cell. Lists of ∼1,500 highly variable genes for the day and the night samples were prepared and used to compute principal components (PC) using RunPCA; the results of PC analysis were projected onto the remaining genes with ProjectPCA (43).

The clustering of cells was done by employing a shared nearest neighbor (SNN)-based algorithm; results were imaged by t-distributed stochastic neighbor embedding (t-SNE) through RunTSNE (parameters: do.fast = TRUE). The 2D projections of the cells generated by this method generates clusters that are color-coded according to FindClusters output. The identity of the clusters were determined using known marker genes. In each sample, the β-pinealocyte population was embedded on the t-SNE plot as a single cluster; it was divided into smaller color-coded clusters by the SNN clustering algorithm. These clusters were consolidated into one large cluster for subsequent study to match the t-SNE embedding. Cellular doublets were eliminated based on expression of moderate-to-high levels of genes that were markers for separate clusters (day, $n = 60$; night, $n = 125$) (see reference (43) for additional details).

NINE CELL TYPES OF THE PINEAL GLAND

Over 5,000 individual cells were subjected to cluster analysis, which detected five major cell types: pinealocytes, astrocytes,

microglia, vascular and leptomeningeal cells (VLMCs), and endothelial cells (**Figure 2A**). The expression of marker genes in these cells confirmed this finding. It was possible to further resolve the cell types according to the results of cluster analysis and marker gene abundance into two pinealocyte subypes (α and β), three subtypes of astrocytes (α, β, and γ), and two subtypes of microglia (α and β). Transcriptomic relationships of the nine cell types as indicated by hierarchical clustering are consistent with our assigned designations (**Figure 2B**).

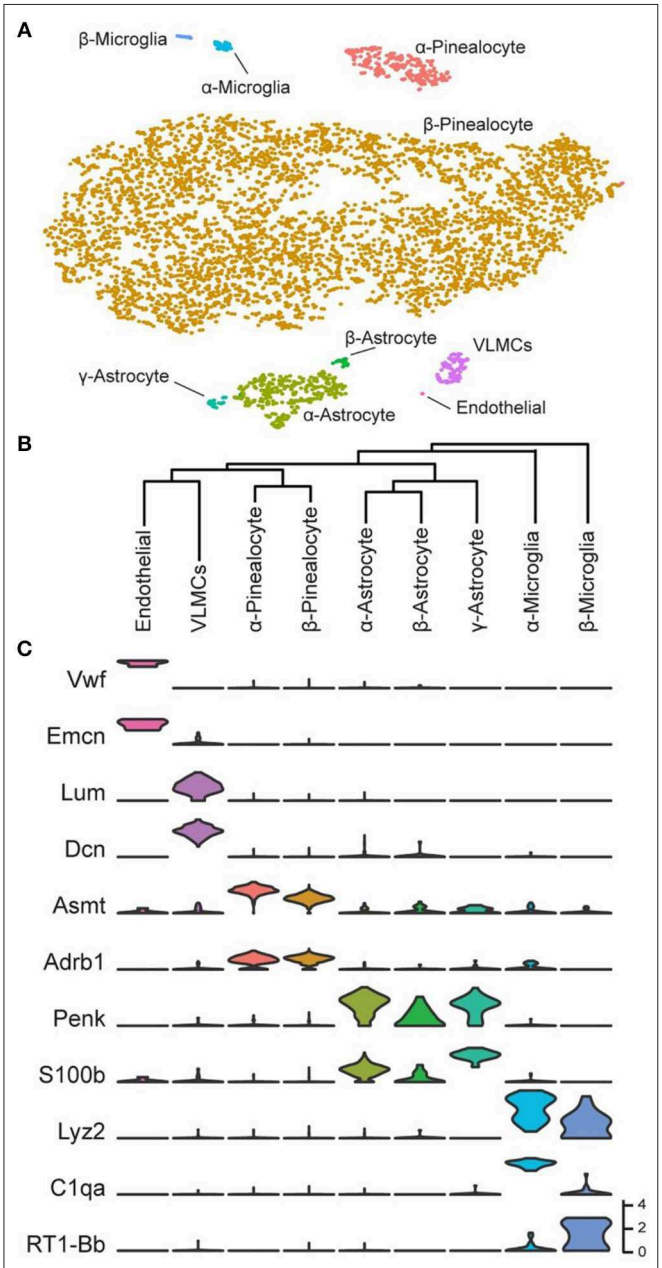

FIGURE 2 | Transcriptomic characterization of cell types in the daytime rat pineal gland. **(A)** t-Distributed stochastic neighbor embedding (t-SNE) visualization of over 5,000 daytime rat pineal gland cells as profiled by scRNA-seq. Cell types are color-coded by cluster. **(B)** Hierarchical clustering dendrogram showing cell type transcriptomic similarity, including two pinealocyte subtypes, the three astrocyte subtypes, the two microglia subtypes, and two vascular-associated cell types: VLMCs and endothelial cells. **(C)** Violin plots of marker genes for cells from each cell type. Y-Axis is natural log of normalized counts. Reproduced from Mays et al. (43). This figure and associated legend is published with permission of the original publisher under license CC0.1.0.

Pinealocytes

The precise proportion of cell-types is difficult to determine with a high degree of confidence because of differences in recovery and cell stability during isolation. However, with this limitation in mind, it appears that 90% of the profiled cells were pinealocytes [Table S1 in (43)], which is generally in line with morphological studies (1, 44) (**Figure 3A**). These cells all expressed high levels of *Tph1, Asmt,* and *Sag* [**Figure 2C**; Figure S1 in (43)]. These cells also expressed high levels of *Gngt1*, *Gngt2*, *Rom1*, *Crx*, *Cngb1*, *Cnga1*, *Pde6c*, and *Slc6a6;* receptors for adrenergic agonists *Adrb1*, *Adra1b*, and *Drd4;* and, receptors for cholinergic agonists *Chrna3* and *Chrnb4* [**Figure 2C**; Figures S1, S2 in (43)]. In addition, these cells expressed a group of 49 transcripts found nearly exclusively in the pineal gland and retina (19) including *Sag* [Figure S1 in (43)], *Gngt1* and *Gngt2* [Figure S4 in (43)], *Crx* and *Neurod1* [Figure S19 in (43)], *Pde6b* [Figure S15 in (43)], *Drd4* [Figure S2 in (43)], and *Cacna1f*, *Cnga1*, and *Cngb1* [Figure S13 in (43)]. The expression of these transcripts exclusively in pinealocytes has not been directly demonstrated previously in most cases; this is because a homogenized mixture of cells in the pineal gland had been used in earlier bulk sequencing

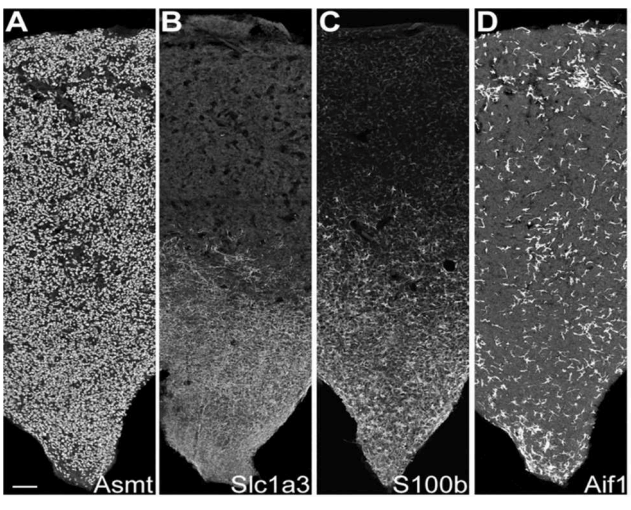

FIGURE 3 | IHC reveals cell type-specific patterns of expression. IHC sections through the rat pineal gland midline; rostral stalk origin at the bottom. The length and middle third of the width of the gland appear. Scale bar = 100 μm. **(A)** Uniform distribution of ASMT-positive pinealocytes. **(B)** Slc1a3-positive γ-astrocytes abundance is greatest in rostral/stalk region. **(C)** S100b-positive cells are abundant in the rostral region; they appear elsewhere with distinctly lower density and weaker expression strength. **(D)** Aif1-positive cells are unevenly distributed throughout pineal gland at low density. See Figure S6 in Mays et al. (43) for full images and further details. Reproduced from Mays et al. (43). This figure and associated legend is published with permission of the original publisher under license CC0.1.0.

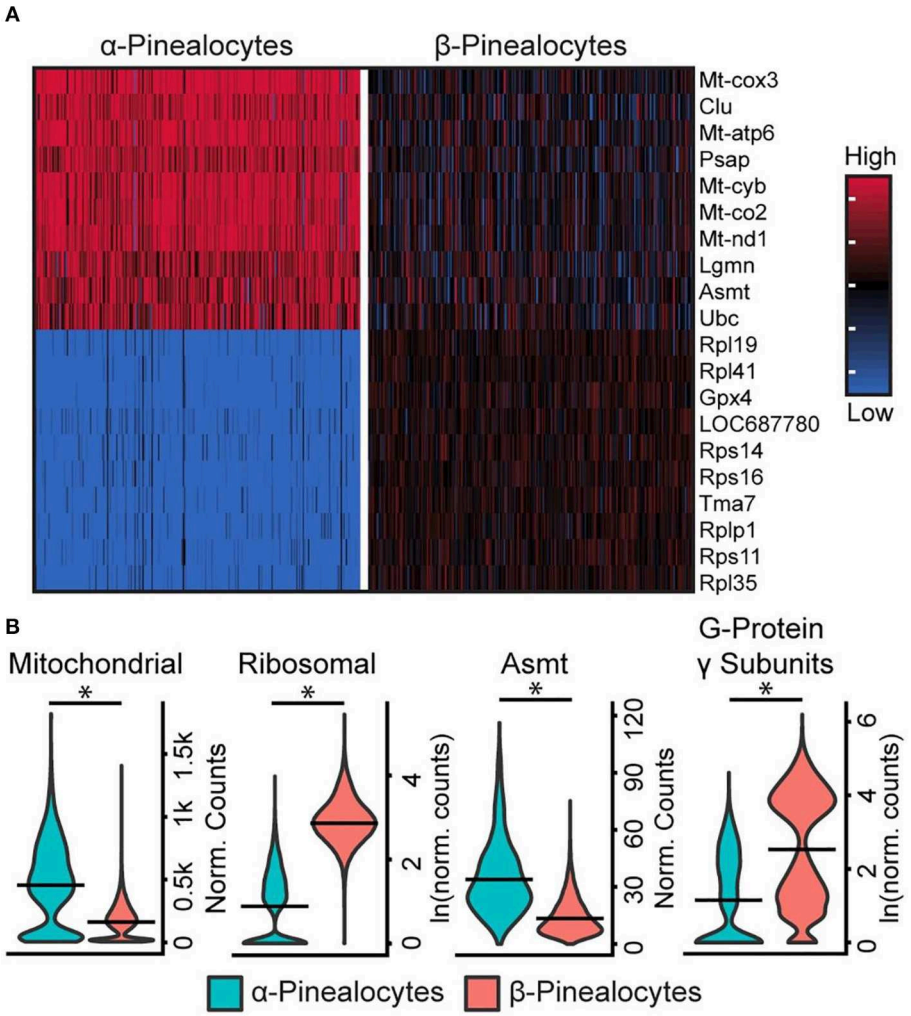

FIGURE 4 | scRNA-seq reveals two transcriptionally distinct pinealocyte populations. **(A)** A heatmap of expression values for the top 10 most differentially expressed genes (by effect size) for α- and β-pinealocytes. Values are Z-scores of counts calculated between all cells of both cell types. Each column represents one cell: random samples of 250 cells per cell type are presented. **(B)** Violin plots of expression distribution differences between two pinealocyte subtypes for three functional groups and one gene, *Asmt*. Y-Axis is either normalized counts or natural log (ln) of normalized counts. Horizontal lines represent the mean. *$p < 0.001$, Wilcoxon rank sum test. All cells from each subtype are included (α-pinealocyte, n = 275; β-pinealocyte, $n = 4{,}822$). Mitochondrial group includes differentially expressed mitochondrial OxPhos genes ($p < 0.05$, $N = 12$, fold change ≥2.0), ribosomal group includes top 20 most differential ribosomal genes by effect size ($p < 0.05$, fold change ≥2.0), G-protein γ-subunits include *Gngt1*, *Gngt2*, *Gng10*, and *Gng13* [see Figure S5 in (43) for individual genes]. Reproduced from Mays et al. (43). This figure and associated legend is published with permission of the original publisher under license CC0.1.0.

studies, precluding the clear association of a gene with a cell type. However, several lines of evidence in the literature point to this conclusion (43).

Five percent of pinealocytes were the α-subtype; the remaining pinealocytes were β-subtypes. Although these two cell types share a characteristic set of marker genes and function as sources of melatonin, analysis revealed some distinct genetic differences and differences in sets of functional groups. The most outstanding included (1) *Asmt;* (2) mitochondrial oxidative phosphorylation (OxPhos) genes; (3) genes that comprise the ribosomal genome; and, (4) G-protein γ-subunits [**Figure 4A**; Figures S1, S3, S4 in (43)]. *Asmt* expression (**Figure 4B**) in α-pinealocytes was 3.4-fold greater, supporting results from previous immunohistochemical studies of ASMT protein (37). The counts for OxPhos and ribosomal transcriptomes were pooled [Figure S5 in (43)] for analysis; α-pinealocytes had a 2.3-fold greater average expression of eight differentially expressed OxPhos genes, and 8.2-fold lower expression of the top 20 differentially expressed ribosomal genes. There also is a 5.4-fold lower average expression of G-protein γ-subunits *Gngt1*, *Gngt2*, *Gngt10*, and *Gng13* in α-pinealocytes relative to β-pinealocytes [**Figure 4B**; Figure S4 in (43)]. The possibility that α-pinealocytes represent stressed cells was rejected because of the opposite and robust differences between the levels of OxPhos and ribosomal genes: the former being higher in α-pinealocytes and the latter higher in β-pinealocytes.

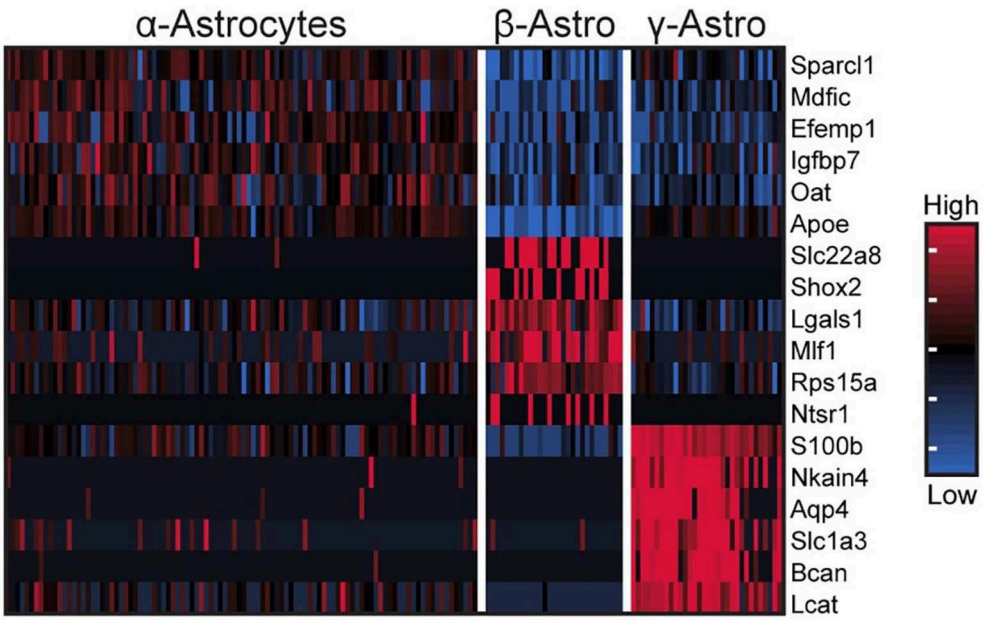

FIGURE 5 | scRNA-seq reveals three transcriptionally distinct astrocyte populations. Heatmap of expression values for the top 6 highest differentially expressed genes (by effect size) for α-, β-, and γ-astrocytes. Values are Z-scores of counts calculated between all cells of the three cell types. Each column represents one cell; a random sample of 100 cells from α-astrocytes are shown; all β- and γ-astrocytes are shown. See also Figure S1 in Mays et al. (43) for more information. Reproduced from Mays et al. (43). This figure and associated legend is published with permission of the original publisher under license CC0.1.0.

Astrocytes

These cells accounted for seven percent of the cells [Table S1 in (43)], based on expression of known markers including *Aldh1a1*, *S100b*, and *Tnfrsf21* [**Figure 2C**; Figure S1 in (43)] (45–47). These cells also highly expressed *Penk*, *Apoe*, and *Esm1* [**Figure 2C**; Figure S1 in (43)]. The percentages of α-, β-, and γ-astrocytes were 85, 7, and 8%, respectively. α-Astrocytes had higher *Sparcl1*, *Mdfic*, *Efemp1*, *Oat*, and *Gad2* expression relative to other subtypes. The β-astrocytes expressed *Slc22a8*, *Shox2*, *Lgals1*, and *Mlf1* at higher levels than other subtypes. γ-Astrocytes were characterized by stronger expression of *S100b*, *Nkain4, Aqp4*, *Slc1a3*, *Bcan*, and *Gfap* [**Figure 5**; Figure S1 in (43)]. Histochemical analysis revealed that γ-astrocytes were primarily limited in distribution to the pineal stalk region, as indicated by Slc1a3 [**Figure 3B**; Figure S6B (43)], as was true of Gfap protein [Figures S6C, S7C in (43)]; this is consistent with previous observations (48–50). *S100b* was expressed in all astrocyte subtypes, but was strongest in γ-astrocytes [**Figure 5**; Figure S1 in (43)]. Detection of S100b-postive cells by IHC revealed astrocytes occur throughout the gland, though higher expression was present in the pineal stalk region, consistent with the higher expression of *S100b* exhibited by γ-astrocytes [**Figure 3C**; Figures S6D, S7D in (43)].

Microglia

One percent of the profiled cells [Table S1 in (43)] were classified as microglia according to expression of *Aif1* and *Lyz2* [**Figure 2C**; Figure S8 in (43)] (45–47). AIF1 IHC Positive cells were present throughout the gland [**Figure 3D**; Figure S6E in (43)]. α- and β-microglia subtypes comprised 64 and 36% of microglia, respectively. These cells were strongly differentiated by complement components *C1qa*, *C1qb*, and *C1qc*, which were high in α-microglia. β-Microglia in contrast had low levels of the complement component transcripts, but high levels of MHC Class II transcripts *RT1-Da*, *RT1-Db1*, and *RT1-Ba* [Figure S8 in (43)].

Vascular Cells

Endothelial cells and VLMCs were detected in low abundance. These cells appear to be in intimate contact, based on expression of *Cdh11 and Gja1* in both [Figure S10 in (43)]. Endothelial cells accounted for 0.1% of cells profiled and were characterized by the expression of *Vwf, Emcn,* and other markers (43).

VLMCs were 2% of the profiled cells, identified by expression markers *Lum, Dcn, Col1a1*, and *Gjb2* [**Figure 2C**; Figure S9 in (43)] (51). VLMCs have never been described in the pineal gland prior to this study. They have the potential of major importance in acting as mediators between circulating signals and pineal cells, in addition to contributing to the extracellular matrix reflecting the expression of collagen and extracellular matrix proteins.

For example, they have receptors for circulating ligands which could act to alter the synthesis and release of secondary signals that impact the function of other cells in the gland. Pineal VLMCs exclusively express *Il13ra2* [Figure S9 in (43)], the transcript that encodes a selective receptor for the cytokine interleukin Il13 (52). Interaction of Il13 with its receptor could impact the pineal gland broadly, perhaps through effects on the extracellular matrix. In addition, vascular cells could act on pinealocytes and astroctyes

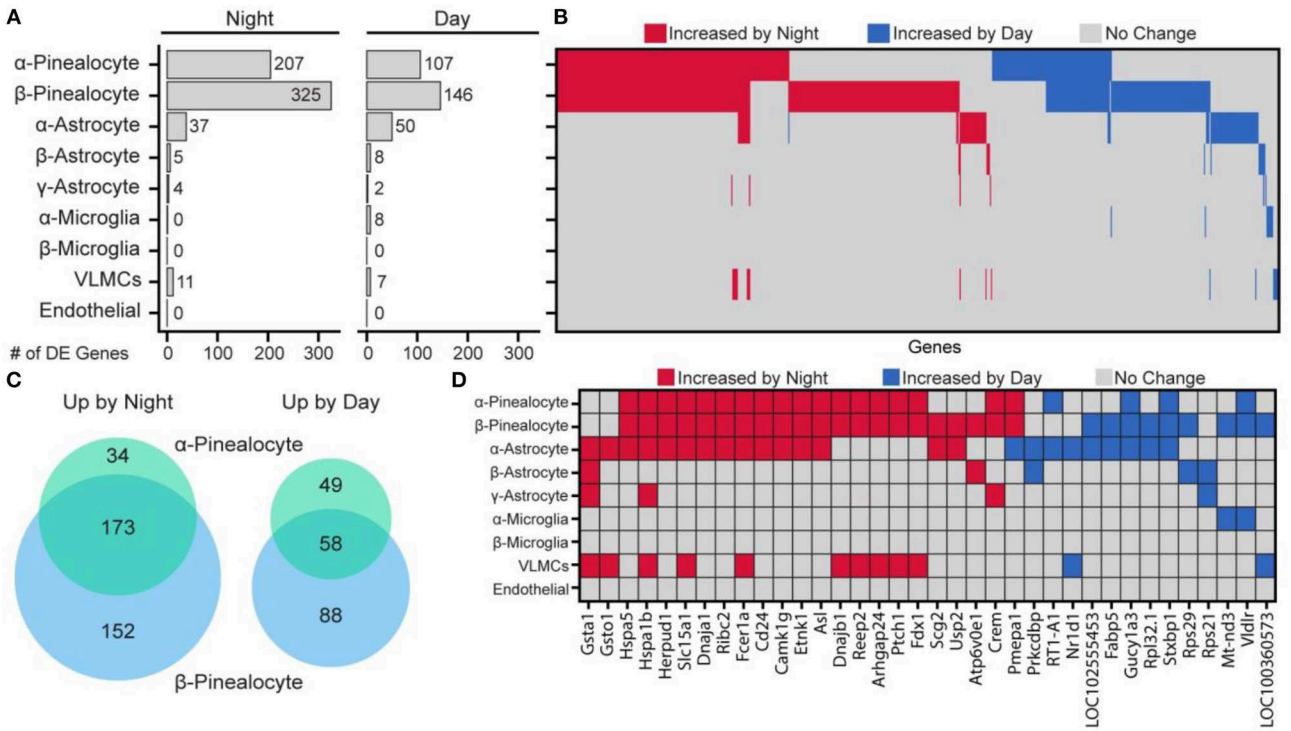

FIGURE 6 | Changes in gene expression between day and night occur in a cell type-specific manner. **(A)** Number of differentially expressed (DE) genes upregulated by night or day by cell type. DE is $p < 0.01$ (Wilcoxon rank sum), when expressed in at least 15% of cells in either of the two samples being tested, fold change ≥ 2.0, and effect size ≥ 0.35. **(B)** Heatmap summary of all 644 DE gene changes by cell type. Each column represents one gene. **(C)** Venn diagram of number of overlapping DE genes in α- and β-pinealocytes by day and night. **(D)** Heatmap summary of DE genes found in at least one non-pinealocyte and one other subtype. See also dot plots in SI of Mays et al. (43). Reproduced from Mays et al. (43). This figure and associated legend is published with permission of the original publisher under license CC0.1.0.

through contact-dependent ephrin ligand-receptor mechanisms (53). Specifically, the ephrin ligand EFNA1 on endothelial cells could bind to the ephrin receptor EPHA4 on pinealocytes; and, the ligand EFNB1 on VLMCs could bind to the receptor EPHB1 on astrocytes (Figure S17 in (43)]. It should also be mentioned that, VLMCs are a standout among cells in the pineal gland for expression of the α_{2A}-adrenergic receptor, encoded by *Adra2a* [Figure S2 in (43)]. Activation of this receptor by circulating norepinephrine or epinephrine can cause catecholamine-induced inhibition of adenylate cyclase and as a result inhibit cyclic AMP dependent processes in the VLMCs. Although of interest, it should be noted that the above are speculations and accordingly require further study to determine their relevance in the context of pineal cell biology.

DAY AND NIGHT CHANGES IN THE PINEAL CELL TRANSCRIPTOME

Day and night expression values in specific pineal cell types were compared. There were considerable differences among the cell subtypes in the number of genes that were differentially expressed between day and night (**Figure 6**). The largest differential expression was found in pinealocytes: 359 genes were upregulated at night and 195 genes were upregulated during daytime. Consistent with prior studies, differentially upregulated genes included *Aanat*, *Crem*, *Drd4*, *Pde10a* (19, 25). Overall, β-pinealocytes had 1.5-fold more genes differentially expressed than α-pinealocytes, with considerable overlap: 173 and 58 of the same transcripts were increased in both subtypes during night and day, respectively (**Figure 6C**). Non-pinealocytes had generally lower day/night differential expression, with the α-astrocytes having 37 genes higher at night and 50 higher during the day. There were relatively fewer differentially expressed transcripts in other non-pinealocytes several of which overlapped between different cell types (**Figure 6D**). The molecular basis of changes in astrocytes is not clear. Whereas, α- and β-adrenergic mechanisms control changes in pinealocytes, the responsible receptors are absent from astrocytes. Other receptors might mediate these changes. Alternatively, the day/night differences could reflect the functioning of an internal clock in these cells, although expression of clock genes is not high [Figure S22 in (43)].

Pinealocytes have a high amount of *Aanat* at night. *Aanat* transcripts were also detected at uniformly low levels in non-pinealocytes [Figure S1 in (43)], probably due to contamination by pinealocyte-derived ambient mRNA. This results in non-pinealocytes erroneously seeming to express *Aanat*

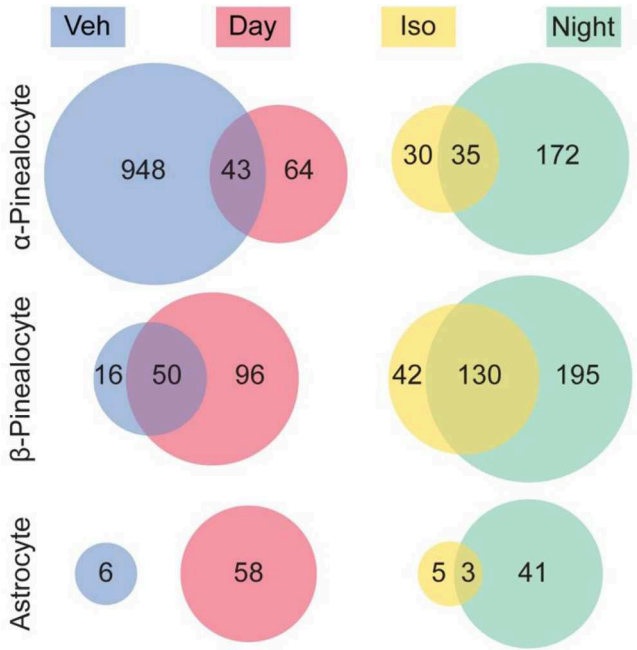

FIGURE 7 | Comparison of differentially expressed genes between the night time pineal gland and isoproterenol-treated pineal gland. Venn diagrams indicate the number of genes that were significantly differentially expressed (DE) in the pineal gland. There was overlap between genes exhibiting DE upregulated at night and by isoproterenol (Iso) treatment, as well as overlap between genes exhibiting DE upregulated during the day and upregulated in the vehicle control treated (i.e., downregulated by isoproterenol treatment), in 3 cell types. Other cell types are not shown. Reproduced from Mays et al. (43). This figure and associated legend is published with permission of the original publisher under license CC0.1.0.

differentially. Because of this, the gene was deleted from non-pinealocyte analysis. *Pmepa1* was determined to be unusual because it was upregulated at night in one cell type (α- and β-pinealocytes) but upregulated during the day in α-astrocytes (**Figure 6D**).

ISOPROTERENOL TREATMENT MIMICS DAY/NIGHT CHANGES IN THE PINEAL CELL TRANSCRIPTOMES OF PINEALOCYTES

It is known that treatment with the β-adrenergic agonist isoproterenol during daytime has similar effects on the pineal transcriptome to those that occur due to neural stimulation at night. It is used in place of norepinephrine because isoproterenol is not taken up into nerve endings in the pineal perivascular space, whereas norepinephrine is rapidly and selectively taken up, thereby largely preventing adrenergic activation (54).

The results of our studies were in line with the interpretation that 97% of the transcriptional changes observed following isoproterenol treatment were in α- and β-pinealocytes (**Figure 7**). This is in agreement with findings of high enrichment with β-adrenergic receptors that mediate night time changes in gene expression. Astrocytes had the remaining 3% of changes, whereas changes in other cells were not detected. It should be noted that upregulation of a similar number of the same genes was observed in isoproterenol-treated glands and in night time glands: upregulation of 54, 76, and 38% of the same genes was seen in α-pinealocytes, β-pinealocytes, and astrocytes, respectively. Four and seventy-six percent of genes suppressed due to isoproterenol treatment in α-pinealocytes and β-pinealocytes, respectively, were also suppressed during the day, that is, they appeared to be upregulated after vehicle control treatment.

IMPLICATIONS

There are broad implications of the findings of scRNA-seq analysis. Several points of interest can be identified, including the selective mechanisms involved in astrocyte gene expression. However, the feature which is especially worthy of additional comment here is the finding of two pinealocyte subtypes. As discussed above, α-pinealocytes are characterized by high levels of *Asmt* and high levels of the mitochondrial genome, and low levels of protein synthesis transcripts and *Gngt1* and *Gngt2*, in contrast to the more abundant β-pinealocytes (**Figure 4B**). Together, they are responsible for the synthesis of melatonin in the pineal gland, with slightly different roles.

It is proposed that the α-pinealocytes are especially highly adapted for the last step in melatonin synthesis. This is supported not only by the high levels of *Asmt* but by the accompanying increase in ATP production by the OxPhos pathway. The main impact on melatonin synthesis is that high ATP leads directly to an increase in SAM, which is synthesized from ATP and methionine (**Figure 8**). Thus, the cells containing both these effects are in a position to methylate N-acetylserotonin at high levels. The focus of the cells on melatonin synthesis is further evidenced by the low levels of protein synthesis enzymes; protein synthesis is the primary consumer of cellular ATP. Adding to this are the low levels of *Gngt1* and *Gngt2*, indicating that G-protein based signal transduction is suppressed; this is in agreement with the finding of lower levels of gene induction in these cells, as discussed above.

The existence of two functionally different pinealocyte subtypes raises the issue of whether α-pinealocytes are compromised to a degree that interferes with the functioning of these cells. This could occur due to relatively lower metabolites and suppressed protein synthesis. One can argue that the absence of some functions, such as maintenance of extracellular matrix, would be compensated for by β-pinealocytes. Also, some essential factors that are reduced in the α-pinealocytes could be provided by the β-pinealocyte. Cell:cell transfer of these factors via gap junctions, membrane permeability, and import/export mechanisms might mediate this export:import function. Also, some proteins may have sufficient stability to prevent a loss of function. Moreover, lowered activity of some processes in the α-pinealocytes may enhance ASMT activity by lowering the production of inhibitors and producing a more favorable biochemical environment for ASMT. Hence, it

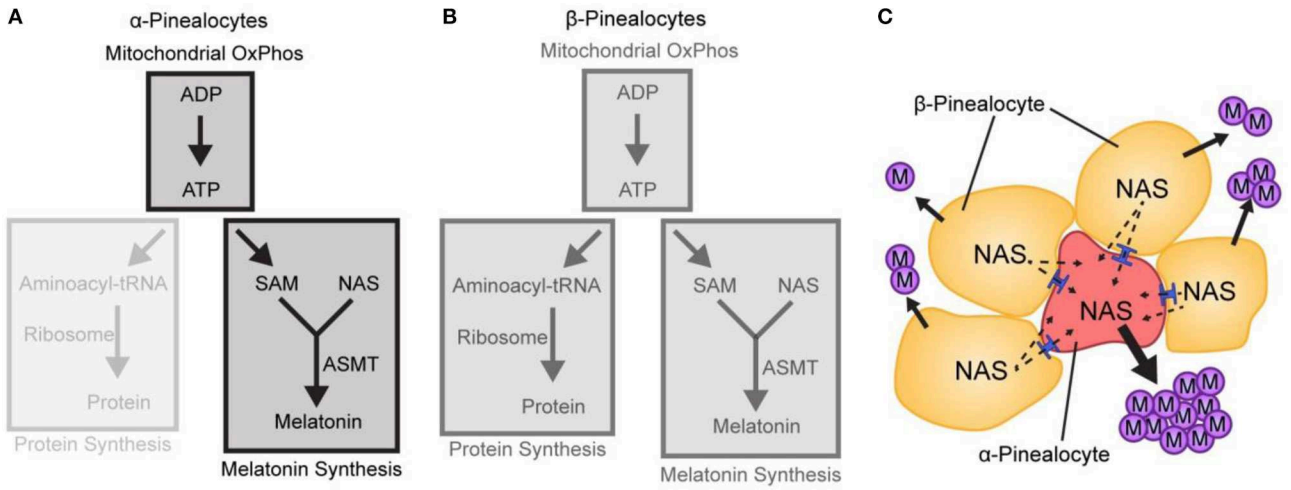

FIGURE 8 | A hypothetical model based on scRNA-Seq depicting differences in melatonin synthesis between α- and β-pinealocytes. **(A,B)** The relative strength of a pathway module is indicated by opacity; greater opacity represents a more active pathway. **(A)** Conversion of N-acetylserotonin (NAS) to melatonin in α-pinealocytes is enhanced by higher ASMT activity and increased S-adenosyl methionine (SAM) availability, which is boosted by greater ATP availability. Increased ATP availability reflects increased ATP production from oxidative phosphorylation (OxPhos); this is inferred by greater expression of mitochondrial genes in α-pinealocytes. ATP availability also results from reduced consumption by protein synthesis, as inferred by decreased ribosomal transcriptome in α-pinealocytes. **(B)** β-Pinealocytes do not have the same enhancements as α-pinealocytes. **(C)** N-Acetylserotonin (NAS) that is not converted to melatonin in β-pinealocytes enters the α-pinealocyte by passive diffusion through membranes and gap junctions (shown in blue) and is converted to melatonin, thereby maximizing melatonin production. Reproduced from Mays et al. (43). This figure and associated legend is published with permission of the original publisher under license CC0.1.0.

seems possible that such changes could support the seemingly compromised α-pinealocytes.

A final issue to be addressed is how the number of each of the pinealocyte subtypes is regulated. One hypothetical possibility is that distinct phenotypes develop early in ontogeny and the cells cannot undergo a shift from one subtype to another. In this scenario, the phenotypes are not reversible; their relative abundance might only reflect selective cell death and replacement. A second hypothetical possibility is that α- and β-pinealocyte phenotypes are reversible and can shift back and forth at any time during development and maturity. The controlling factor or factors could be circulating in nature or reflect neural stimulation, perhaps influenced by the day length. This hypothetical reversible mechanism might fine tune melatonin production.

CONCLUDING COMMENT

The work reviewed here is impressive in documenting how methods have evolved from requiring a few milligrams of tissue to document a single transcript to documenting thousands of transcripts in a single cell!

scRNA-seq establishes a new foundation for research on pineal cell biology by introducing new methods and concepts and by segregating gene expression into separate cells. Moreover, it has reshaped our thinking about the pineal gland by adding to the complex nature of the tissue, by providing transcriptionally defined cell types. The work is unique in that two states of physiological activity—day and night—are characterized, which adds another dimension to the value of scRNA-seq of this tissue.

Work on the pineal gland has the potential to improve our understanding of the basic mechanisms that underlie the function of this tissue in non-human primates and humans. Bulk sequencing of the rhesus pineal gland indicates that there are fundamental differences between it and the rat, as regards day/night changes in transcript abundance (22, 55). It will be of interest to use scRNA-seq technology to learn more about the human and rhesus pineal glands, with the intention of understanding how cells in this tissue communicate and are regulated.

One avenue that will challenge investigators is the analysis of isoform regulation (23) on a single cell basis, with the goal of understanding the association of specific isoforms with cell types and how they are regulated. The discoveries revealed by scRNA-seq and advanced forms of sequencing will shape future studies on pineal cell biology.

AUTHOR CONTRIBUTIONS

SC, CF, SH, LH, JMa, MCK, MWK, JMu, MR, LS, and DK: substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data for the work. SC, SH, LH, JMu, MCK, MWK, JMa, MR, and DK: drafting the work or revising it critically for important intellectual content. SC, LH, SH, MWK, JMa, MR, JMu, MCK, MR, and DK: provided approval for publication of the content.

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REVIEW
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Melatonin Target Proteins: Too Many or Not Enough?

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The neurohormone N-acetyl-5-methoxytryptamine, better known as melatonin, is a tryptophan derivative with a wide range of biological effects that is present in many organisms. These effects are believed to rely either on the chemical properties of melatonin itself as scavenger of free radicals or on the binding of melatonin to protein targets. More than 15 proteins, including receptors (MT_1, MT_2, Mel1c, CAND2, ROR, VDR), enzymes (QR2, MMP-9, pepsin, PP2A, PR-10 proteins), pores (mtPTP), transporters (PEPT1/2, Glut1), and other proteins (HBS, CaM, tubulin, calreticuline), have been suggested to interact with melatonin at sub-nanomolar to millimolar melatonin concentrations. In this review we assemble for the first time the available information on proposed melatonin targets and discuss them in a comprehensive manner to evaluate the robustness of these findings in terms of methodology, physiological relevance, and independent replication.

Keywords: melatonin, GPCR, QR2, ROR, PR-10, MMP-9, PEPT1/2, Glut1

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INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is an evolutionary ancient molecule that is synthesized by uni- and multicellular organisms, ranging from bacteria, protists, fungi, macroalgae, plants, and animals. Melatonin has been associated with many physiological functions that evolved along the evolutionary time scale (1). Melatonin is a tryptophan derivative that due to its hydrophobicity can cross membranes by passive diffusion (2). The passive diffusion is believed to occur in pinealocytes, specialized cells of the pineal gland in vertebrates that release melatonin during the night, immediately after its circadian synthesis (3). An additional chemical property of melatonin shown *in vitro* is its antioxidant ability by scavenging free radicals (4). This antioxidant property has been proposed to be the most primitive function of melatonin being relevant along the evolutionary time scale from unicellular organisms, to plants and vertebrates. This aspect has been reviewed in another article of this series (1) and will not be addressed in this article.

Here we will focus on the mechanisms of action of melatonin, more specifically, on those effects that are mediated by its binding to molecular targets. Due to the cell-membrane penetrating properties of melatonin, extra- as well as intracellular proteins were considered as potential melatonin targets since the beginning. Over the years more than 15 different proteins have been proposed to bind melatonin ranging from receptors, enzymes, pore proteins, transporters, and various other proteins (**Table 1**). Examples of "functional interactions" that are often indirect, i.e., through regulation of gene transcription, including recently discussed examples such as calpain or SIRT3 will not be addressed here (38).

TABLE 1 | Characteristics of melatonin target proteins.

Protein family	Melatonin target	Affinity/Efficacy for melatonin	Effect of melatonin	Direct binding of melatonin				References	Reviews
				YES/NO	Methodology	Type of binding site	Independent replication (14)		
Receptor	MT1	0.1 nM (Kd) (1)	Activation	YES	Ligand binding, co-crystal structure	Orthosteric (co-cyrstal, pharmacol.)	YES	(5, 6)	(7, 8)
	MT2	0.1 nM (Kd) (1)	Activation	YES	Ligand binding, co-crystal structure	Orthosteric (co-cyrstal, pharmacol.)	YES	(6, 9)	(7, 8)
	Mel1c	1 nM (Ki) (2)	Activation	YES	Ligand binding	Orthosteric (pharmacol.)	YES	(10, 11)	
	CAND2	10 nM (Ki) (2)	Activation	YES	Ligand binding	Unknown	NO	(12)	
	ROR/RZR	5 nM (Kd) (3)	activation	YES	Ligand binding	unknown	unsuccessful	(13, 14)	(15)
	VDR	20 μM (Kd) (4)	Increases affinity of Runx2 for VDR	YES	Isothermal titration calorimetry	The C-terminal ligand binding domain (LBD) of the VDR	NO	(16)	
Enzyme	QR2	1 μM (Kd) (4)	Inhibition	YES	Ligand binding, isothermal titration calorimetry, co-crystal structure	Catalytic site (co-crystal)	YES	(17, 18)	(19)
	MMP-9	50–100 μM (IC50) (5)	Inhibition	YES (12)	Docking studies, gelatin zymography assay	Catalytic site (docking)	NO	(20)	
	Pepsin	10 μM (Kd) (4)(6)	Unknown	YES	Isothermal titration calorimetry, equilibrium microdialysis	Catalytic site (docking)	NO	(21)	
	PP2A	Unknown	Suppression of PP2A inhibitor effect (11)	YES (12)	Docking studies	Near the catalyt sites (docking)	NO	(22)	(23)
Transporter	PEPT1/2	0.5–1 mM (Km) (7)	Transport of melatonin into cells and mitochondria	YES (12)	Docking studies	Substrate site (docking), competion with classical substrates	NO	(24)	
	GLUT1	Unknown	Transport of melatonin into cytoplasm, mitochondria	YES (12)	Docking studies	Substrate site (docking), competion with classical substrates	NO	(25)	(26)
	Hyp-1	Unknown (low affinity)	Binding of melatonin (11)	YES	Co-crystal strcuture	Binding site (2 sites) (co-crystal)	NO (13)	(27)	
	LLPR-10.2B	Unknown (low affinity)	Binding of melatonin (11)	YES	Co-crystal strcuture	Binding site (2 sites) (co-crystal)	NO (13)	(28)	
Others	mtPTP	0.8 μM (IC50) (8)	Inhibition of open propability	YES (12)	Electrophysiology	Unknown	NO	(29)	

(Continued)

TABLE 1 | Continued

Protein family	Melatonin target	Affinity/Efficacy for melatonin	Effect of melatonin	Direct binding of melatonin				References	Reviews
				YES/NO	Methodology	Type of binding site	Independent replication (14)		
	Serum albumin	10 μM (Kd) (4)(9)	Binding	YES	Ligand binding, isothermal titration calorimetry, absorption spectroscopic	Binding site	YES	(30, 31)	
	CaM	>2 mM (Kd) (10)		YES	Fluorescence spectroscopy, NMR and molecular dynamics studies (recomb. protein)	Binding site	YES	(32, 33)	
		1 nM−1 μM (IC50) (5)	Inhibition	YES (12)	Docking studies, enzyme activity	Binding site at CaM in complex with effectors	YES	(34–36)	
	Calreticuline	1 nM (Kd) (3)	Unknown	YES	Ligand binding	Unknown	NO	(37)	

(1) [3H]-MLT saturation binding; (2) [125I]-MLT competition binding; (3) [125I]-MLT saturation binding; (4) isothermal titration calorimetry; (5) enzyme activity inhibition; (6) Equilibrium microdialysis; (7) transport into cells; (8) inhibition of opening of mPTP; (9) Absorption spectroscopy; (10) Fluorescence spectroscopy, NMR; (11) hypothetical; (12) suggested; (13) shown for another member of the PR-10 protein family; (14) refers to the replication of the key results (binding of melatonin to targets) by another group in an independent article.

The proposed direct targets of melatonin will be discussed regarding the half-maximal effective concentrations (EC_{50}) of melatonin for the referred effects, the affinity of melatonin for the binding sites (K_d), and the way melatonin binds to these sites, by resuming any available information on these aspects. The robustness of the proposed targets will be evaluated in terms of independent replication, a criteria that is routinely used in other fields, for example, to confirm the successful deorphanization of G protein-coupled receptors (GPCRs) (39). Finally, we will discuss how the measured EC_{50} and K_d values of melatonin for the different targets match with melatonin concentrations reported in different organisms, organs and biological fluids under physiological and pathological/stress conditions.

RECEPTORS

Receptors have been among the first suspected molecular targets of melatonin (5, 9, 10). Structural similarity of melatonin, in particular to serotonin and dopamine, and the sensitivity of melatonin-induced pigment aggregation in *Xenopus* dermal melanophores to the $G_{i/o}$ protein inhibitor pertussis toxin pointed toward 7-transmembrane-spanning GPCRs as likely candidates for melatonin receptors (40). The cell-penetrating properties of melatonin inspired the search for additional, intracellular, melatonin receptors (**Table 1**).

GPCRs

GPCRs are currently the best-characterized melatonin targets and are found in invertebrates and vertebrates. These receptors are classified into three groups called MT_1 (previously Mel1a), MT_2 (Mel1b) and GPR50 (in mammals), or Mel1c (in non-mammals) (5, 9, 10). All these receptors bind melatonin with high affinity (0.1–1 nM) (7) with the exception of GPR50, the mammalian ortholog of Mel1c that lost its ability to bind melatonin during the evolutionary divergence of the therian lineage of mammals from the monotremes (11, 41, 42). Melatonin is considered to be the natural agonist of these receptors that promotes G protein activation and beta-arrestin recruitment. These results have been replicated by many groups. Extensive pharmacological profiles have been established for these receptors with melatonin and also with various synthetic agonistic and antagonistic compounds. In addition, polymorphisms of the MT_1 (43–45) and MT_2 (46–49) receptors have also been identified, some of which affect the binding and signaling properties of these receptors, being factors known to influence both disease risk and/or be of pharmacogenetic relevance (8, 50). Progress on these aspects is regularly updated by the International Union of Basic and Clinical Pharmacology (IUPHAR) melatonin receptor subcommittee (7, 8, 51). Pharmacological studies have been recently complemented by crystallization studies of human MT_1 and MT_2 receptors co-crystallized with several melatonin analogs in their inactive states (**Figures 1A,B**) (52, 53). Both receptors show a high degree of amino acid homology [55% overall and 70% within the transmembrane (TM) domains], and a similar, shallow, melatonin binding pocket located within the TM domains (**Figures 1A,B**). The binding pose of the melatonin

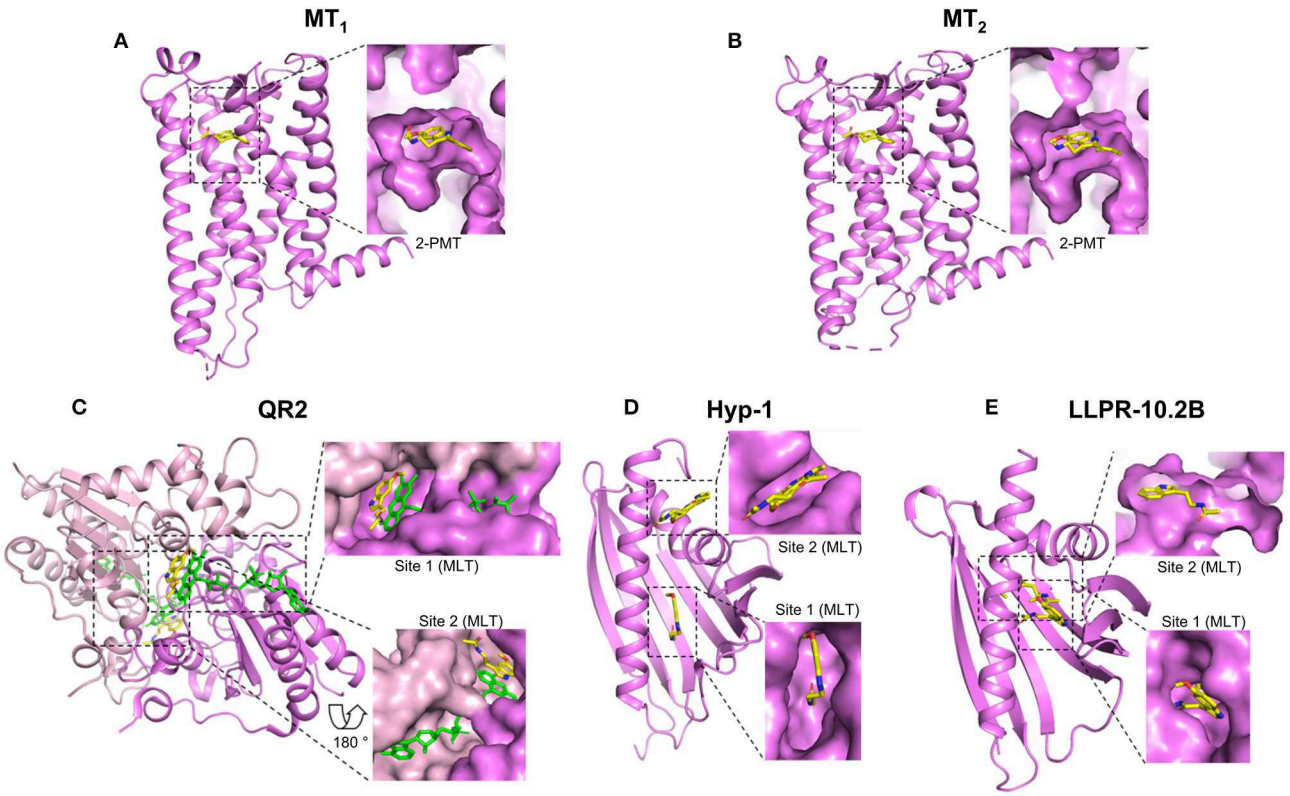

FIGURE 1 | Crystal structures of melatonin target proteins in complex with melatonin or close derivatives. The full scales of the proteins are shown in cartoon in violet and the bound ligands in yellow. The ligand binding sites are highlighted by dashed rectangles and the details are shown aside by enlarged surface areas of the proteins. For those located inside, sliced views are shown to visualize the ligand. **(A)** MT_1: MT_1 receptor, PDB 6ME3; **(B)** MT_2: MT_2 receptor, PDB 6ME6; **(C)** QR2: Quinone reductase 2, PDB 2QWX, the second monomer is in light pink; FAD cofactors are shown in green **(D)** Hyp-1: St. John's wort Hyp-1 protein, PDB 5I8F; **(E)** LLPR-10.2B: Yellow lupin LLPR-10.2B protein, PDB 5MXB. MLT: Melatonin; 2-PMT: 2-phenylmelatonin. Structural views were generated using the PyMOL Molecular Graphics System (Schrodinger LLC), based on available information from the references mentioned in the text.

derivative 2-phenylmelatonin (2-PMT) proved to be very similar for both receptors with identical key residues, including the participation of the extracellular loop 2 (ECL2) ($N^{4.60}$, F^{ECL2}, Q^{ECL2}, and $N^{6.52}$) (superscripts represent Ballesteros–Weinstein nomenclature; **Figures 1A,B**). Interestingly, the binding pocket in the MT_1 structure has one lateral ligand entry channel (from the membrane environment), whereas two ligand entry channels, the lateral one, and an additional one from the extracellular side, are visible in the MT_2 structure (52–54). These different ligand entry channels as well as their different widths and differences in the overall volume of the pockets with the pocket of MT_2 being about 50 $Å^3$ larger than that of MT_1, offer future opportunities for subtype selective drug development.

Recently, the protein product of the *CAND2* gene from *Arabidopsis thaliana* was proposed to fold into 7-transmembrane domains and to bind the radiolabeled 2-[^{125}I]iodomelatonin and melatonin with high affinity (K_d = 0.7 nM and K_i ~ 10 nM, respectively) (12). This recent study has not been replicated independently for the moment. This "phytomelatonin" receptor seems to be totally unrelated to the mammalian melatonin receptors as it shows no significant overall amino acid sequence similarity (around 10%) nor does it contain any key amino acid residue known to be part of the melatonin binding pocket in melatonin receptors from animals. A pharmacological characterization of this melatonin binding site is still needed in order to compare it with mammalian melatonin receptors.

Nuclear Receptors

Due to the cell-penetrating property of melatonin, the existence of intracellular receptors has been considered from the beginning. In this context, nuclear melatonin targets attracted much attention. This culminated in the claim that melatonin binds to and activates the retinoic acid receptor-related orphan receptor-β (RORβ), a member of the subfamily of retinoid receptors (13). However, these results could not be reproduced by other groups and were retracted later (14). In 2014, the natural ligands for ROR were identified to be sterols and oxysterols including cholesterol and its derivatives, all structurally very different from melatonin (55, 56). Despite this compelling negative evidence, it is unfortunate that numerous publications still interpret their melatonin effects by binding of melatonin to RORs and that these interpretations are repeated in many review articles. The current status on nuclear melatonin receptors has been summarized recently by Hardeland, reaching the conclusion that effects of melatonin on nuclear receptors are likely to be rather indirect (15). More recently, the Vitamin D receptor

(VDR), a nuclear receptor, has been reported to bind melatonin directly with a K_d of 21.2 ± 1.9 μM (16). The authors found that melatonin binds to the ligand binding domain (LBD) located in the C-terminus of VDR. The binding of melatonin to VDR facilitates the interaction of VDR with the transcription factor Runt-related transcription factor 2 (Runx2), thus promoting the transcriptional activity of Runx2 indirectly. These results need to be replicated in the future but re-open clearly the long-standing discussion on the possible existence of nuclear melatonin targets.

ENZYMES

Several enzymes, like the quinone reductase 2, metalloprotease-9, pepsin, and protein phosphatase 2 have been proposed to bind melatonin directly (**Table 1**). There is no apparent similarity between these proteins and their proposed binding sites, and melatonin concentrations necessary for binding vary widely.

Quinone Reductase 2 (QR2)

QR2 is most likely the best-characterized melatonin target apart from G protein-coupled melatonin receptors (19). The chase for this melatonin target started in 1988 with the identification of the MT3 (also known as ML2) melatonin binding site that was clearly different from the GPCR binding sites (17). This binding site was confirmed independently by several groups. In 2000, 5-methoxycarbonylamino-N-acetyltryptamine (MCA-NAT), a melatonin derivative with high affinity (nM) for the MT3 binding site and only modest affinity for MT_1 and MT_2 was used for affinity purification of MT3. The only protein retained was QR2 (57). Expression of QR2 was sufficient to replicate the pharmacology of the MT3 binding site and co-crystals showed that melatonin binds indeed to the catalytic site of QR2 (18) (**Figure 1C**). Using purified QR2, the K_d for melatonin was 1 μM in isothermal titration calorimetry (ITC) experiments and the inhibitory capacity of melatonin was in the range of 10–130 μM, depending on the functional assay used to measure QR2 activity (19). The MT3-specific pharmacological profile was recapitulated with purified QR2. The published crystal structure shows that QR2 is a symmetric dimer with two melatonin binding pockets (**Figure 1C**) at the interface of the two QR2 monomers (18). The poses of the two bound melatonin molecules are similar but not identical in both pockets. Hydrophobic interactions/contacts between melatonin and the protein as well as the π interactions between the FAD co-factor, such as the parallel stacking of the melatonin indole moiety on top of the FAD isoalloxazine ring and the melatonin benzene ring on top of the FAD piperazine-like moiety are important elements. QR1 belongs to the drug metabolism enzymes for which the plasticity of the catalytic site is believed to be an inherent property to accommodate natural and synthetic xenobiotic compounds from the environment. A similar broad substrate specificity can be hypothesized for QR2 which evolved from QR1 (58). QR2 has been proposed to be a membrane-associated protein although its presence as a soluble enzyme has also been claimed (19). In conclusion, although there remain some controversies about the subcellular localization of QR2, this enzyme most likely corresponds to the MT3 binding site that binds melatonin in the low μM range.

Metalloproteinase-9 (MMP-9)

MMP-9 is located in the extracellular matrix (ECM) and contributes to ECM remodeling by cleaving various ECM components. Melatonin has been shown to negatively regulate MMP-9 expression through various mechanisms (see below). An alternative explanation has been proposed based on results obtained with purified MMP-9, which showed an inhibitory effect of melatonin in the gelatin zymography assay with an IC_{50} of 50–100 μM, suggesting a direct interaction of melatonin with MMP-9 (20). This hypothesis is compatible with molecular docking studies showing that melatonin can bind to a small cleft of MMP-9 that corresponds to its catalytic site (20). Interactions of melatonin with the key residues of the catalytic site of MMP-9 including the three zinc-coordinating histidines were suggested (20). This provides a reasonable explanation for the inhibitory effect of melatonin. MMP-9 has also been co-crystallized with other high-affinity indole-based inhibitors such as phosphinate and carboxylate derivatives (K_i = 10–200 nM) (59). Molecular docking studies with melatonin suggest direct binding of melatonin to MMP-9 but with lower affinity than phosphinate and carboxylate derivatives, most likely because of differences in the pose of the indole rings (20, 59). An overall protective effect of melatonin in MMP-9-dependent experimental injury models is observed in several studies including in ethanol- (60) and indomethacin-induced (61) gastric cancer, experimental colitis (62), global cerebral ischemia (63), and in Blood-Brain Barrier permeability (64). The high melatonin concentrations necessary for these effects, either in cellular models (50–1,000 μM) or *in vivo* (10–100 mg/kg), are compatible with the IC_{50} of melatonin for purified MMP-9, but raise the question of whether MMP-9 inhibition occurs under physiological conditions or whether it represents a purely experimentally-induced effect.

Pepsin

Pepsin is a protease that is released in the stomach to catabolize ingested proteins into peptides. The active binding site of pepsin is located in a cleft between the N- and C-terminal domain with two aspartate residues, Asp32 and Asp215, which are important for its enzymatic activity. A recent biophysical study reported the direct interaction of melatonin with the catalytic site of pepsin with Asp32 being part of the suspected binging pocket (21). Binding occurred at a 1:1 stoichiometry and with a K_d of 10 μM as determined by titration calorimetry and equilibrium microdialysis with recombinant pepsin. Melatonin and pepsin can be both found in relatively high concentrations in the gastro-intestinal tract (see below) but their putative relationship is currently unknown. Independent replication of these results has not been reported for the moment.

Phosphoprotein Phosphatase 2A (PP2A)

PP2A is a member of the Ser/Thr phosphatases subfamily. Inhibition or down-regulation of PP2A promotes hyperphosphorylation of neuronal proteins like Tau, followed by neuronal cell death and neurodegenerative diseases. Melatonin and its derivatives have been reported to be protective in this context (65). Several hypotheses have been put forward to explain the effect of melatonin, including the anti-oxidant activity of

melatonin and a putative direct interaction of melatonin with PP2A. The latter hypothesis was fueled by the observation that gramine derivatives, which are structurally related to melatonin, suppress the inhibitory effect of okadaic acid on the enzymatic activity of PP2A (22). Docking studies indicated the possible binding of these gramine derivatives near the catalytic site of PP2A (22). A similar scenario was proposed for melatonin in a recent review article (23). Taken together, in the absence of experimental evidence for direct binding of melatonin to PP2A and for an effect of melatonin on the enzymatic activity of PP2A, this enzyme remains a hypothetical melatonin target.

TRANSPORTERS

Transport proteins help to transport molecules across membrane barriers like the plasma membrane or membranes of intracellular compartments, either passively or actively (against a concentration gradient with an energy cost). Due to its lipophilic properties, melatonin is believed to rapidly distribute all over the body through passive diffusion (2, 66). However, differences in the tissue and cellular distribution of melatonin might suggest additional regulated uptake mechanisms (26). Two proteins, the glucose transporter GLUT1 and the oligopeptide transporters PEPT1/2, have been recently proposed to transport melatonin across plasma and mitochondrial membranes (**Table 1**). In addition, two plant proteins, Hyp-1 and LLPR-10.2B, belonging to the pathogen-response-10 (PR-10) protein family, have been shown to bind and possibly transport melatonin (**Table 1**).

Glucose Transporter 1 (GLUT1)

Along this idea of assisted transport of melatonin across membranes, Glut1 was recently proposed to be involved in cellular melatonin uptake (25). GLUT1 levels are particularly high in erythrocytes and also found in the brain, the blood-brain barrier and other tissues. Pharmacological inhibition of GLUT1 and competition of glucose uptake by high melatonin concentrations (mM), together with molecular docking studies on XylE, an *Escherichia coli* homolog of GLUT1-4 transporters, suggest that melatonin binds to GLUT1 at a site that overlaps with glucose binding (25). This interesting study will need independent replication and confirmation of direct binding of melatonin to GLUT1. A more detailed characterization of the transport capacity at melatonin concentrations below the mM range is warranted to appreciate the full physiological relevance of this proposed transport mechanism.

Oligopeptide Transporter 1/2 (PEPT1/2)

The oligopeptide transporters PEPT1 and PEPT2 are responsible for the uptake of small peptides and peptide-like molecules in the intestine, kidney, and brain. Ectopic expression occurs also in tumors. A recent report shows that PEPT1/2 can improve the basal uptake of melatonin in cells (K_m = 0.5–1 mM) when applied at 50 μM concentration (24). Uptake was competed by several known PEPT1/2 substrates and docking studies suggested interaction of melatonin with key amino acid residues of the binding domain of these transporters. PEPT1/2-dependent melatonin uptake was measurable into whole cells and isolated mitochondria. PEPT1/2-dependent and PEPT1/2-independent uptakes were equally fast in reaching an equilibrium within 2–3 min, suggesting that the primary impact of PEPT1/2 would be an increase in melatonin uptake capacity of cells at micromolar to millimolar melatonin concentrations. These recent results on PEPT1/2-dependent melatonin uptake have not been replicated independently for the moment. The impact of the PEPT1/2-dependent uptake at low melatonin concentrations remains elusive. Intriguingly, expression of isoform 2 of PEPT1/2 is restricted to pinealocytes, with a pronounced circadian rhythmicity in its expression (100-fold upregulation during the dark phase), suggesting a putative role on the regulation of melatonin synthesis by a so far poorly characterized feedback mechanism (67).

PR-10 Proteins

Plants are known to produce melatonin (68). Stress evokes a number of defense responses in plants including the expression of specific genes that encode pathogenesis-related (PR) proteins. Members of the PR-10 subclass are structurally characterized by a so-called PR-10-fold and are believed to bind small-molecule mediators, such as plant hormones. For two PR-10 proteins, Hyp-1 and LLPR-10.2B, the crystal structures in the presence of melatonin have been solved (27, 28) (**Figures 1D,E**). The structures of Hyp-1 and LLPR-10.2B are similar, both assemble the baseball-glove grip shape by a large seven-stranded antiparallel β-sheet over a long variable C-terminal helix, as well as the two well-defined melatonin binding sites. However, the mode of melatonin binding and the residues that participate in ligand docking are quite different in these two proteins, as the shapes of their binding cavities are not identical (**Figures 1D,E**). For Hyp-1, binding site 1 is located in an internal cavity of the baseball-glove grip shape and binding site 2 within the external cleft that forms around the V-shaped fork of two α-helices. Crystal structure data suggest that melatonin could have two alternative binding modes (see two melatonin molecules positioned in site 2) (**Figure 1D**). For LLPR-10.2B, both binding sites are within the internal cavity of the baseball-glove grip shape (**Figure 1E**), and the external melatonin binding site (site 1), not the deeper one (site 2), can be competed by trans-zeatin, a well-characterized PR-10 binding protein.

FURTHER MELATONIN TARGETS

A range of other melatonin target proteins have been proposed and studied in more or less detail (**Table 1**).

Serum Albumin

Early studies showed that melatonin and other methoxyindoles reversibly bind to a high capacity, low affinity binding site in plasma (30, 69). Fractionation studies of plasma proteins and *in vitro* studies with purified plasma proteins identified this binding site as serum albumin (30). Quantitative methods such as isothermal titration calorimetry and absorption spectroscopic with purified albumin revealed a 1:1 (melatonin: albumin) stoichiometry and a binding constant (Ka) of 1×10^5 L mol^{-1} (31). Albumin fulfills thus the criteria for an efficient carrier

protein with high binding capacity and low affinity to transport significant amounts of the carrier without interfering with its biological activity.

Mitochondrial Permeability Transition Pore (mtPTP)

The mtPTP is a multi-protein complex found at the contact site between the inner and outer mitochondrial membrane. Under conditions of oxidative stress, high Ca^{2+} and low ATP levels, a number of proteins including Bax and Bad are recruited and enable the pore formation at its high conductance state, resulting in the release of Ca^{2+} into the cytosol. Recording of the mtPTP channel currents from patches of the inner mitochondrial membrane showed a concentration-dependent inhibition of mtPTP currents by melatonin ($IC_{50} = 0.8\,\mu M$) (29). These electrophysiological data indicate a direct effect of melatonin on the mtPTP complex. This effect could contribute to the reported anti-apoptotic effects of melatonin, in particular under conditions of transient brain ischemia. This interesting study was not replicated nor followed up for the moment. No more information is available about the identity of the precise melatonin target candidate of the mtPTP complex, which is composed of more than 10 proteins.

Calmodulin (CaM)

CaM is a highly conserved Ca^{2+} binding protein that regulates a large number of Ca^{2+}-dependent signaling events. Studies with various biological sources containing CaM suggested high-affinity binding of melatonin to CaM ($K_d = 0.2$–$1\,nM$) (34, 35). Subsequent fluorescence spectroscopy, NMR, and molecular dynamics studies with purified CaM confirmed the Ca^{2+}-dependent binding of melatonin to CaM, but in a much lower affinity range ($Kd > 2\,mM$) (32, 33). The interaction occurs presumably through one of the hydrophobic binding pockets of CaM, which is exposed on the protein surface upon the Ca^{2+}-induced conformational changes. The huge difference (six orders of magnitude) in apparent affinity of melatonin for purified CaM vs. biological samples remains unexplained. Docking studies of melatonin to the Ca^{2+}-CaM-CaM-kinaseII (CaMKII) complex suggest an improved affinity of melatonin for CaM in CaM-effector complexes (36). This is compatible with the observation that the Ca^{2+}-CaM complex undergoes an additional conformational change upon interacting with CaM effector proteins. Further support for the importance of CaM effector proteins in melatonin binding to CaM comes from several studies reporting an inhibitory effect of melatonin on the enzymatic activity of CaM effectors such as phosphodiesterases (PDE) (IC_{50} ~1 nM) (70), neuronal Nitric-Oxide Synthase (nNOS) (IC_{50} ~1 μM) (71, 72) and CaMKII (IC_{50} ~10 nM) (73). The shallow concentration-response curves, spanning 5–6 orders of magnitude, suggest an indirect effect of melatonin on the enzyme activity. For nNOS, the non-competitive behavior and the fact that CaM antagonists, Ca^{2+} chelators and an excess of CaM abolish the effect of melatonin on its activity argue for CaM being the primary melatonin target protein of nNOS inhibition (72).

Binding of melatonin to microtubules has been suspected very early on (74) and subsequently characterization suggests that at nanomolar concentrations, the cytoskeletal effects of melatonin could be mediated by the Ca^{2+}-CaM complex, while at higher concentrations (10 μM) "non-specific" binding of melatonin to tubulin occurs (75). These studies were not followed further and the precise nature of the melatonin target protein(s) (tubulin, CaM, other…) remains to be independently confirmed. Alternatively, signaling initiated by G protein-coupled melatonin receptors could be also responsible for the rearrangement of cytoskeleton proteins (76, 77).

In summary, despite the fact that CaM was among the first melatonin target proteins discovered, the nature of this interaction and its importance are still not clearly defined. Apparent affinities vary widely in the literature, the affinity for the purified Ca^{2+}-CaM complex is low (mM range) and the interesting hypothesis of high-affinity binding of melatonin to CaM in Ca^{2+}-CaM effector complexes is waiting for direct experimental validation.

Calreticulin

Calreticulin, a ubiquitous and highly conserved Ca^{2+}-binding protein that has chaperon activity and controls intracellular Ca^{2+} homeostasis, has been purified by melatonin affinity chromatography from nuclear extracts from rat hepatocytes. 2-[^{125}I]iodomelatonin binding studies with recombinant GST-tagged calreticulin revealed the high-affinity binding of melatonin ($K_d = 1\,nM$) that was dependent on Ca^{2+} and not competed by NAS, 4P-PDOT or luzindole, three G protein-coupled melatonin receptor ligands (37). This biochemical study qualifies calreticulin as a melatonin target candidate that merits independent replication.

EMERGING FEATURES OF MELATONIN BINDING TO ITS TARGETS BASED ON CRYSTAL STRUCTURES

Melatonin can be divided into three parts based on its chemical structure: the methoxy side chain, the middle indole ring (consisting of a benzene ring fused to a pyrrole ring) and the alkylamide side chain. Among all the published crystal structures of protein complex with melatonin or its close derivatives (**Figure 1**), the indole ring, in particular the benzene ring, is always involved in the melatonin-protein interaction through hydrophobic contacts or π interactions (**Table 2**). Similarly, the alkylamide side chain often interacts with target proteins (through hydrophobic contacts/hydrogen bonds), with the exception of one of the two bound melatonin molecules in QR2 (**Table 2**, **Figure 1C**). In contrast to the alkylamide side chain, the methoxy side chain does not make contacts with the target proteins in most of the cases, with the exception of the high-affinity MT_1 and MT_2 receptors (**Figures 1A,B**). This feature, together with the relatively small binding pocket and ligand entry channel, most likely define the structural basis for high-affinity binding of melatonin to these targets (52, 53). The binding preference of target proteins for the alkylamide chain

TABLE 2 | Structural elements of melatonin involved in the interactions with its target proteins.

Receptor	PDB	Ligand	Affinity	Methoxy		Benzene ring		Pyrrole ring		Alkylamide		
				Position	HB	HC	Pi	HC	Pi	Position	HB	HC
MT1	6ME3	2-PMT	nM	Flat	X	X				Down	X	
MT2	6ME6	2-PMT	nM	Flat	X	X				Down	X	X
QR2	2QWX	Melatonin (site 1)	μM	Flat		X	X		X	Up		
		Melatonin (site 2)		Flat		X	X			Down		X
Hyp-1	5I8F	Melatonin (site 1)	mM	Down		X	X		X	Down		X
		Melatonin 1 (site 2)		Down		X	X			Down		X
		Melatonin 2 (site 2)		Flat		X	X			Up	X	
LLPR-10.2B	5MXB	Melatonin (site 1)	mM	Down		X		X	X	Down	X	
		Melatonin (site 2)		Flat		X		X		Up	X	X

HB, Hydrogen Bonds; HC, Hydrophobic Contacts; Pi, Pi (π) Interactions.

might be explained by the high flexibility of this part in respect to the indole ring, thus providing several options for interactions with different proteins ("up" and "down" positions in **Table 2**). The short methoxy chain apparently prefers to stay within the same plane formed by the indole ring ("flat" position in **Table 2**) and rotate to the same side like the alkylamide chain in some cases ("down" position in **Table 2**).

MELATONIN CONCENTRATIONS

Taken together, more than 15 melatonin target proteins have been proposed (**Figure 2**) that bind melatonin at very different concentrations—from subnanomolar to millimolar concentrations. This raises the question of whether such a huge range of melatonin concentrations exists to be sensed by the different target proteins. The answer to this question is not trivial since, apart from plasma melatonin levels, there has been a lot of debate about melatonin levels in various organs and organisms, as discussed below briefly.

In Non-vertebrates

The capacity for melatonin synthesis can be observed in all major taxa studied so far, including bacteria, dinoflagellates and other eukaryotic protists, macroalgae, plants, fungi, and various groups of invertebrate animals. Melatonin concentrations reported suggest important differences between taxa, with some studies reporting values in the upper micromolar range (26, 78). This domain suffers from a lack of data replication in the strict sense since studies are rarely performed under identical conditions, as different sources of biological material (different species, locations of collection and environmental conditions, etc.) and methods of sample preparation, melatonin extraction, and melatonin dosage are used.

In plants, more specifically in *Arabidopsis thaliana*, melatonin is believed to protect against abiotic stress through its antioxidant properties and through its action as plant hormone (68). The protein encoded by the *CAND2* gene in *Arabidopsis thaliana* has been recently shown to be a G protein-coupled receptor for melatonin that regulates stomatal closure through a H_2O_2 and Ca^{2+} signaling pathway (12). Interestingly, K_i values for melatonin are in the range of 10 nM, which are of high affinity and likely to be reached under physiological and/or stress conditions. The importance of melatonin in plants is further supported by genetic manipulation of the genes of melatonin biosynthesis as upregulation of melatonin synthesis yields improved tolerance abilities, enabling plants to better survive under hostile environmental conditions (79). The physiological relevance of melatonin binding to PR-10 proteins, the other plant proteins reported to bind melatonin, remains elusive as K_d values are in the mM range.

Significant melatonin levels in plants and other sources raise the question of the impact of dietary sources of melatonin (80). Many studies report melatonin content of ∼1 ng/mL, however big variations are reported as well (81, 82). Several studies reported the impact of dietary melatonin from fruits on human serum melatonin levels (83), however the effects remained modest and should be also considered in light of the endogenous production of melatonin in the gastrointestinal tract (GIT) and its absence of contribution to circulating melatonin levels (see below).

In Vertebrates

Plasma melatonin levels vary considerably between different animals and even on an individual level. In addition, melatonin levels can be altered under certain conditions, as demonstrated in humans. Indeed, there is a decline in melatonin levels with age and under several diseases, meaning that the establishment of a reference for melatonin concentration is not straightforward. In mammals including humans melatonin is produced in a circadian manner with plasma daytime melatonin levels around 5 pg/mL or less and nighttime levels rising up to 100–150 pg/mL (∼0.65 nM) (84). At this concentration range, the only confirmed melatonin targets are the GPCRs, MT_1, MT_2, and Mel1c (**Figure 2**). Potential candidates are calreticulin (single study still awaiting independent confirmation) and CaM-effector complexes (still awaiting experimental validation of docking predictions). In vertebrates, the pineal gland has been identified as the primary site of rhythmic melatonin synthesis that determines plasma melatonin levels. Pineal melatonin is

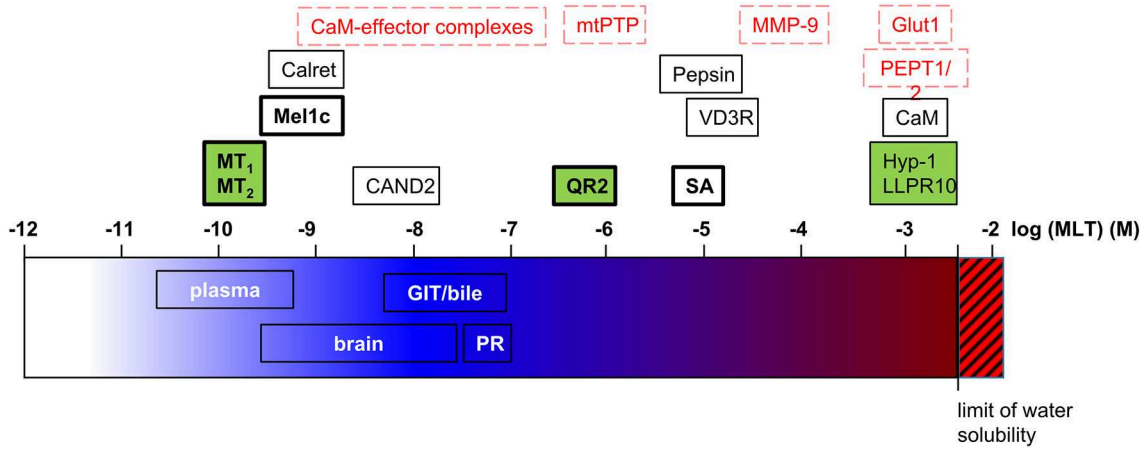

FIGURE 2 | Melatonin target proteins and melatonin concentrations. Melatonin target proteins are positioned on the log scale of molar melatonin concentrations according to their affinity/efficacy for melatonin. The range of melatonin concentrations measured in biological fluids and tissues are indicated on the same scale. Legend for melatonin target proteins: black letters, demonstrated direct targets; red letters, suggested direct targets (docking, functional studies); bold letters, findings were independently replicated; green background, co-crystal structures with melatonin or phenyl-melatonin available. Calret, calreticulin; GIT, gastrointestinal tract; MLT, melatonin; PR, pineal recess; SA, serum albumin.

released in the peripheral circulation via the vein of Galen in the cerebrospinal fluid (CSF) directly via the pineal recess (PR), an evagination of the third ventricle in contact with pinealocytes. In the PR, nighttime melatonin concentrations of 20,000 pg/mL (90 nM) have been detected in sheep (85). Melatonin is then spread over the brain through the CSF from which it penetrates into the different tissues generating a melatonin gradient from the periventricular to the most distal cerebral tissues ranging from 17 to 0.35 nM at night (86). Interestingly, whereas melatonin levels vary in a circadian manner in periventricular tissues, like in the plasma, the variation was only marginal in brain tissues more distal from ventricles. The melatonin concentrations of 300 pM measured at these locations would be still sufficient to activate MT_1 and MT_2 receptors. Taken together, even though the highest melatonin levels close to the pineal gland are up to 100 times higher (up to 90 nM) compared to plasma levels, no obvious additional melatonin targets to the abovementioned ones have been identified so far (**Figure 2**). The three candidates getting the closest in terms of melatonin affinity/efficiency are QR2, VDR, and mtPTP (1–20 μM) [see (87) for review on QR2]. Since melatonin levels reported above are mean values, it cannot be excluded at present that higher melatonin levels can be reached locally, in specific sub-regions and on the subcellular level (see below). In addition, the highly diffusible nature of melatonin, being released right after its synthesis, also makes it difficult to estimate its concentration at a specific location at a given time.

Apart from the pineal gland, several other sources of melatonin synthesis have been described in vertebrates (26). The retina is the second tissue where melatonin production follows a circadian rhythm, similarly to the pinealocytes. Melatonin production from all other extrapineal sources is not rhythmic and does not contribute to the plasma levels of melatonin. Maybe the highest reported extrapineal melatonin levels are in the bile and in enterochromaffin-like cells of the GIT reaching levels that are 10–100 times higher than plasma levels (up to 50 nM) (88–91). Why these significant sources of melatonin do not contribute to circulating melatonin levels remains unclear. Considering local effects of melatonin in these organs, it can be assumed that reported melatonin levels bind a similar repertoire of potential melatonin targets as detailed above for the CSF and the brain. Whether melatonin levels measured in the GIT are sufficient to bind to pepsin (K_d = 10 μM) in the stomach remains to be demonstrated.

Extrapineal melatonin production has also been reported from immune cells where it occurs in an inducible manner. For example, mononuclear cells from human blood activated by zymosan or by *Escherichia coli* produce melatonin in the order of hundreds of pg/mL (∼1 nM), which in turn modulates the phagocytic activity of these cells by an autocrine action mediated by melatonin receptors (92–94). Human lymphocytes, rodent peritoneal macrophages, bone marrow-derived dendritic cells and the macrophage cell line RAW 264.7 also have been reported to produce melatonin in response to diverse stimuli, including lipopolysaccharides from bacteria, serum from tumor-bearing animal models, and adrenergic stimulation (92, 95–98). Similar to what was previously mentioned about melatonin from the GIT, it is also not clear why melatonin produced by these cells does not impact the overall circulating level of melatonin.

A significant number of people take exogenous melatonin. In the USA, an estimated 3.1 million adults (1.3% of the adult population) take melatonin on a daily basis (99). Melatonin is popular for the promotion of improved sleep initiation and fast adjustment in situations of circadian misalignment (such as jet-lag when traveling over several time zones), but also as a prophylactic anti-aging treatment and as a preventive

treatment for neurodegenerative diseases and cancer. Typical doses of melatonin range from 0.3 to 10 mg per day (100). At a dose of 0.3 and 2 mg of melatonin, plasma peak levels increase 2 to 3 times over endogenous peak levels, respectively (84, 101). In critically ill patients with a reduced disappearance rate of melatonin, as well as in normal healthy subjects, administration of 3 and 5 mg was reported to increase plasma peak levels 7 to 15 times reaching levels of ∼50–100 nM (102, 103). It can be therefore anticipated that maximal serum peak levels of melatonin will not reach far beyond 100 nM even upon treatment with melatonin, which is ∼200 times higher than endogenous peak levels and in the range of melatonin levels reported in the brain and GIT/bile. Taken together, exogenous administration of melatonin increases plasma peak level up to 200 times, but is unlikely to reach μM or mM concentrations to bind to additional, low-affinity target proteins *in vivo*.

At the Subcellular Level

Several reports suggest that melatonin might be differentially distributed in subcellular compartments. In particular, cell nuclei and mitochondria seem to contain higher melatonin concentrations than other compartments such as the cytosol. Side-by-side comparison of melatonin and serotonin using amperometric and fluorescence measurement methods in intact cells demonstrated that extracellular melatonin, but not serotonin, equilibrates within seconds with the cytoplasm confirming that melatonin crosses biological membranes rapidly (2). Other studies suggest facilitation of melatonin transport, in particular into mitochondria, through PEPT1/2 and GLUT1 transporters (26). Recently, mitochondria isolated from neurons have been proposed to synthesize melatonin but the levels reached are unknown (104). The relative contribution of pineal melatonin synthesis to mitochondrial melatonin levels in neurons, i.e., whether melatonin is imported in or exported out of mitochondria and whether this occurs by passive diffusion or through the proposed PEPT1/2 and GLUT1 transporters, remain interesting questions to be solved in the future. The presence of melatonin in mitochondria is not only of interest because of the presumed elimination of free radicals by the antioxidant action of melatonin but also because of the presence of MT_1 receptors in mitochondrial membranes coupled to the inhibition of cytochrome c release and apoptosis (104, 105).

Similar to mitochondria, nuclei have been proposed to contain melatonin targets. As detailed before, its nature remains to be determined in light of the inconclusive evidence for RORβ and follow-up studies on VDR will show the robustness of this recently proposed nuclear melatonin target. The presence of MT_1 or MT_2 receptors in the nuclear membrane cannot be completely ruled out either in analogy to other GPCRs with nuclear localization. Alternatively, calreticulin present in ER membranes could be also of relevance due to the close spatial proximity of the ER membrane and the nuclear envelop. Altogether, the subcellular distribution of locally produced melatonin and its targets are still an active and challenging object of study in the melatonin field. Progress in this field holds great promise to solve much of the mystery regarding the mismatch between melatonin concentrations required to bind to melatonin target proteins and the *in situ* concentrations measured so far.

CONCLUSION

Currently 18 different melatonin targets have been proposed comprising receptors, enzymes, transporters and other proteins (**Table 1**). Surprisingly, 12 of them are still awaiting independent replication. The level of melatonin to which these targets respond range over 7 orders of magnitude, from sub-nanomolar to millimolar concentrations (**Figure 2**). Only 8 of the validated/proposed targets respond to low to moderate melatonin levels. For 5 melatonin targets, structural information is available from co-crystals. These targets provide first insights on the structural requirements for melatonin binding as they bind melatonin with high (nM), medium (μM), and low (mM) affinity concentrations. More studies will be necessary to validate the proposed targets by independent replication. Pharmacological profiles will have to be established similar to what has been done for melatonin receptors already starting back in 1975 (106). Further studies will also be necessary to determine local melatonin production and melatonin concentrations with more precision, directly at their targets in specific cellular environments and in intracellular compartments, to judge the relevance of melatonin and its targets with μM and mM affinity/efficacy. Development of non-invasive detection methods will be beneficial in this respect to capture the real levels of the highly diffusible melatonin. The authors hope that this review will provide a rational basis for a consensus of validated melatonin target proteins and help to eliminate ungrounded claims about melatonin targets, in particularly in the review literature of the melatonin field.

AUTHOR CONTRIBUTIONS

RJ initiated the review and wrote the first draft together with LL and EC. The initial literature research was performed by NL. All the authors participated in the editing of the manuscript and in the preparation of the figures and tables. LL generated structural models shown in **Figure 1** and defined structural requirements of melatonin binding shown in **Table 2**.

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Melatonin and Female Reproduction: An Expanding Universe

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For more than a half century the hormone melatonin has been associated with vertebrate reproduction, particularly in the context of seasonal breeding. This association is due in large measure to the fact that melatonin secretion from the pineal gland into the peripheral circulation is a nocturnal event whose duration is reflective of night length, which of course becomes progressively longer during winter months and correspondingly shorter during the summer months. The nocturnal plasma melatonin signal is conserved in essentially all vertebrates and is accessed not just for reproductive rhythms, but for seasonal cycles of metabolic activities, immune functions, and behavioral expression. A vast literature on melatonin and vertebrate biology has accrued over the past 60 years since melatonin's discovery, including the broad topic of animal reproduction, which is far beyond the scope of this human-focused review. Although modern humans in the industrialized world appear in general to have little remaining reproductive seasonality, the relationships between melatonin and human reproduction continue to attract widespread scientific attention. The purpose of this chapter is to draw attention to some newer developments in the field, especially those with relevance to human fertility and reproductive medicine. As the vast majority of studies have focused on the female reproductive system, a discussion of the potential impact of melatonin on human male fertility will be left for others.

Keywords: melatonin, reproduction, human, fertility, pregnancy, gonads, uterus

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BRIEF INTRODUCTION

By virtue of being a small, amphiphilic, indoleamine molecule, melatonin (5-methoxy-N-acetyl tryptamine) is synthesized *de novo* from serotonin (5-hydroxy-tryptamine), a highly dispersed biologically active molecule in its own right. Historically, melatonin has been considered an endocrine hormone released from the epithalamic pineal gland, which then acts on specific G-protein-coupled melatonin receptors in target tissues of both adults and the fetus (1, 2). More recently, melatonin has been reported to be synthesized in small amounts by a wide variety of animal cells and tissues as well as diverse organisms, including all kingdoms of living organisms [cf. (3, 4)], where it presumably has local paracrine and autocrine actions, some of which are probably independent of specific melatonin receptors (5). Indeed, melatonin has been reported to interact with a great many cellular proteins, including enzymes, channels, transporters, signaling molecules, etc [for a recent comprehensive review, see (6)]. Thus, melatonin is perhaps best defined as both a pineal hormone and a bioactive amine with cellular targets near its site of synthesis in some tissues.

While such generalizations permit the inclusion of many effects, it doesn't remove a number of challenges for the interpretation of the research data involving melatonin. For example, with regard to targets, the reported affinities of the two known human melatonin receptors (in both cell

expression systems and *ex vivo*) are in the nanomolar range [cf. (7)], whereas many if not most experimental protocols have employed very pharmacological concentrations to achieve significant effects. Another point to consider is that although plasma melatonin levels are physiologically elevated for many hours at nighttime, protocols often expose tissues or cells to only very short melatonin treatments, which may be physiologically irrelevant. A common response to these concerns is that local concentrations may be quite high and/or constant—especially if there is local constitutive melatonin synthesis. Recent studies suggest that melatonin synthesis by mitochondria may be important for subcellular physiological processes (8). However, there is little experimental evidence that disruption of such local melatonin production has meaningful cellular consequences [in contrast to the removal of plasma melatonin via extirpation of the pineal gland, which has numerous effects on the reproductive axis of laboratory animals - cf. (9)].

The human being is exposed to varying levels of melatonin from conception to death. Much like the hormones thyroxine, insulin or cortisol, the molecule melatonin has a variety of diverse roles to play as a function of developmental life stage (embryo, neonate, adolescent, or adult). It seems likely that many of these actions of melatonin could be permissive or synergistic (like the aforementioned hormones), but there is remarkably scant research into this distinct possibility. A significant step in this direction are the findings of circadian clock gene regulation by melatonin in several tissues of the reproductive axis in both the embryo and adults.

Melatonin has often been described as a chemical output signal of the central circadian oscillator (the hypothalamic suprachiasmatic nuclei, SCN). The clearest support for this statement is the abolition of plasma melatonin rhythmicity following disruption of the neural connectivity between the SCN and the pineal gland. As mentioned earlier, the nocturnal melatonin signal duration is of major importance for physiological seasonality, however, the circadian phasing of the melatonin signal has important ramifications for general circadian functions, including body temperature, endocrine rhythms, and sleep (10). This is in part due to fact that melatonin receptors are expressed in the SCN and can mediate phase-shifting feedback effects of melatonin. Hence, in clinical studies of melatonin actions on the reproductive (or any) system, it is imperative to keep issues such as the timing of melatonin administration (day vs. night) and the duration of the plasma melatonin levels following exogenous melatonin administration in mind. Unfortunately, these considerations are too often overlooked in clinical trials involving melatonin treatments.

Especially in the area of reproductive biology, it is clear that the physiology of animal models is often not comparable to the human state, most especially with regard to ovulatory cycles and the regulation of pregnancy [cf. (11)]. Hence, promising results from melatonin experiments in other species need to be confirmed in clinical trials before one can draw any conclusions of relevance to reproductive medicine. The most obvious historical case in point is the controversy in the late decades of the twentieth century regarding melatonin as an "anti-gonadotrophic" or a "pro-gonadotrophic" hormone (in the human it is physiologically neither, although at high doses there may be some inhibitory effect on ovulation—see more below).

Human reproduction is a challenging object of study for obvious reasons, e.g., population heterogeneity, ethical limits when experimenting with humans, high research costs and appropriate technologies, etc. The quality of clinical data—its statistical power, reproducibility, appropriateness of controls, treatment variations, and so on—make the attainment of firm conclusions thus far about melatonin's normal physiological role in human reproduction difficult. Similarly, validation of proposed pharmacological uses for melatonin or analogs in the treatment of puberty, infertility, menopause, etc has not yet been achieved due to limited published scientific literature. The goal of this chapter is stimulate future research into the relationship of melatonin to human reproductive function in anticipation of generating novel diagnostic and therapeutic tools to improve human health and fertility.

MELATONIN AND PUBERTY

Clinical reports from "pre-melatonin" days (i.e., prior to Aaron Lerner's discovery of the hormone in 1958) identified a potential link between human puberty and pineal tumors. For example, early in the twentieth century Marburg [see his (12) review]—based on reported clinical findings of pineal tumors in children—developed the hypothesis that secretions of the pineal inhibit human reproductive activity. Indeed some clinicians of that generation used pineal extracts to treat precocious puberty (13).

Following the seminal studies of Wurtman et al. (14)—who demonstrated antigonadal effects of melatonin in female rats—the investigation of melatonin's impact on mammalian reproduction rapidly expanded [cf. (15)]. However, with regard to human puberty and its regulation by melatonin, conflicting reports appeared in the later quarter of the twentieth century. Whereas, some groups found higher plasma melatonin levels associating with prepubertal and delayed pubertal conditions (16, 17) and inversely lower levels of melatonin after puberty or in cases of precocious puberty (18–20), numerous other groups found no significant differences between normal and disordered puberty (21–24). These discrepancies have led to skepticism among twenty-first century clinicians regarding the importance of melatonin in normal pubertal development. In both young males and females, the puberty-related decline of high childhood melatonin levels has been correlated more to advancing Tanner stages than to chronological age (25), but no clear causative basis for this relationship has been established for humans.

In view of the circadian secretion of melatonin and the circadian nature of pituitary hormone levels during puberty and in adults, it has long been suggested that melatonin regulates human reproductive cycles. The pulsatile release of GnRH and hence gonadotropin pulse frequency is highest during the night during puberty (26) and the monthly surge of LH and FSH secretion at ovulation also occurs mainly during the latter hours of the dark phase (27, 28). To what extent the temporal coincidence of hypothalamic secretions with melatonin release simply reflects coordinated downstream activation of neural

pathways under control of the central circadian oscillator in the SCN as opposed to explicit regulation of the neuroendocrine axis by melatonin remains unclear—most likely both pathways play some role.

MELATONIN AND THE FEMALE REPRODUCTIVE CYCLE

Melatonin receptors have been demonstrated in a variety of cell types in the female reproductive tract uterus. As shown in **Table 1** the majority of studies demonstrate dual expression of both MT1 and MT2 receptors. Hence, one should consider all of these cells to be potential targets for melatonin action.

Some 30 years ago, Brzezinski et al. demonstrated that human preovulatory follicular fluid contained melatonin at levels higher than plasma melatonin levels (44). This was subsequently confirmed and later shown to vary inversely with day length and concomitantly with follicular progesterone (P4) levels (45, 46), suggesting preferential uptake of circulating melatonin by the ovary. Nakamura et al. (47) subsequently found that larger preovulatory follicles had higher melatonin levels than smaller immature follicles. Later observations that increasing oral doses of melatonin results in significantly elevated melatonin concentrations in follicular fluid of women volunteers (48) also support this view. Several reports followed in which melatonin was shown to modulate progesterone production by cultured human granulosa/luteal cells (29, 49, 50). More recently, the effects of melatonin on cultured human granulosa/luteal cells has been extended to include synergism with hCG—albeit at very high melatonin concentrations (51).

Interestingly, when combined with progesterone, melatonin at high doses is able to suppress human ovulation (52). As will be discussed later, rising progesterone levels during human pregnancy (when ovulation is strongly suppressed) are accompanied by rising plasma melatonin levels. It could be insightful to assess the effects of progesterone on melatonin receptor expression in the human ovary and other reproductive tissues.

TABLE 1 | Melatonin binding sites in the human female reproductive system.

Organ	Cells	Receptor type	Response	References
Ovary	Granulosa/luteal	nd	↑ P4	(29–31)
		MT1, MT2	↓ P4	(32, 33)
		nd	↓ BMP6 signaling	(34)
		nd	↓ oxid. Stress	(31)
Uterus	Myometrium	MT1, MT2	↑ contractility	(35, 36)
				(37)
Breast	Glandular epithelium	MT1	↓ERα transcriptional activity	cf. (38)
Placenta	Choriocarcinoma	MT1, MT2	↓hCG secretion	(39)
	Trophoblast	MT1, MT2	↓Inflammation, autophagy	(40, 41) (42, 43)

Among 61 women undergoing assisted reproductive therapy (ART) treatment cycles it was reported that a positive correlation exists between follicular melatonin levels and markers of ovarian reserve, e.g., anti-Muellerian hormone and baseline FSH levels (53). These authors also found a similar correlation between follicular fluid melatonin levels and *in vitro* fertilization (IVF) outcomes and oocyte quality. Similarly, Zheng et al. (54) found a significant positive correlation between follicular fluid melatonin concentrations and antral follicle count in women undergoing *in vitro* fertilization—also consistent with a supportive or protective action of melatonin on ovarian cycle progression.

These results have motivated a number of studies into the potential benefit of pharmacological melatonin supplementation in the treatment of infertility [cf. (55) for review]. Although the etiology of infertility is complex and not fully clarified, a recurring aspect appears to be excessive production of reactive oxygen species in the follicular fluid (56). In an oft-cited study by Tamura et al. (57), it was reported that when patients were given 3 mg of melatonin orally in the evening from the fifth day of the previous menstrual cycle until the day of oocyte retrieval, intra-follicular concentrations of melatonin rose 4-fold. Markers of intra-follicular oxidative damage were decreased after melatonin treatment compared to those in the prior cycle, suggesting that melatonin treatment reduces intra-follicular oxidative stress. These investigators then assessed the clinical outcomes of 115 patients who failed to become pregnant in the previous IVF-ET cycle with a low fertilization rate ($< 50\%$). In the 56 patients treated with melatonin, the fertilization rate ($50.0 \pm 38.0\%$) was markedly improved compared with the previous IVF-ET cycle ($20.2 \pm 19.0\%$), and 11 of 56 patients (19.6%) achieved pregnancy. In contrast, in the 59 control patients, the fertilization rate (22.8 ± 19.0 vs. $20.9 \pm 16.5\%$) was not significantly changed, and only 6 of 59 patients (10.2%) achieved pregnancy. These intriguing findings are consistent with the view that pharmacological melatonin administration increases intra-follicular melatonin concentrations, reduces intra-follicular oxidative damage and may have a beneficial effect on fertilization and pregnancy rates during ART. In a recent report, similar benefits were found in 40 women with idiopathic infertility who were administered pharmacological melatonin and who subsequently showed improved intrafollicular oxidative capacity and oocyte quality in IVF protocols (58).

These data point to a potential benefit of melatonin in the process of oogenesis. In this regard, the high levels of melatonin required for effects seem consistent with high follicular fluid concentrations of melatonin. Some investigators have also proposed that follicular granulosa cells have the capability for local melatonin synthesis (59, 60), which if confirmed would add new insight into melatonin's role as a paracrine modulator in the reproductive system of humans.

Many female reproductive hormones undergo 24-h rhythms under both standard sleep-wake cycles and under constant routine conditions, indicating that they are under endogenous circadian control (61, 62). Interestingly, these rhythms are robust in the early follicular phase but not in the luteal phase of the menstrual cycle, which is largely under the control of high luteal progesterone secretions. Perturbations of the human

circadian system (e.g., from shift work) are known to disrupt reproductive cycles [cf. (63) for review]. However, data are lacking on the potential role of melatonin to the etiology of these disruptions, despite initial early findings in one small study (64) that demonstrated a high incidence of irregular menstrual cycles in night workers whose melatonin levels were significantly suppressed.

MELATONIN AND PREGNANCY

Following earlier research on maternal transfer of melatonin to the fetus and neonatal development of pineal melatonin rhythmicity in the 1980s and 1990s (65–67), a period of relative quiescence welcomed the transition to a new millennium. However, a growing number of studies in the past decade have focused on the action of melatonin on the placenta and fetal development as well as circadian roles for melatonin before and after parturition.

It is now relatively well-established that a prime entrainment signal from the maternal circulation to fetus is melatonin (68), which crosses the placenta (69), and can bind to melatonin receptors in numerous fetal tissues (70).

Melatonin receptor (MT1 and MT2) transcripts and proteins have also been detected in human placentae (39–41) and subsequently shown to be expressed throughout pregnancy, albeit with declining levels after the first trimester (71). In addition, mRNA and protein expression of the melatonin-synthesizing enzymes, AANAT and HIOMT, have also been reported (40, 41), leading to the view that in addition to the rising plasma melatonin levels during pregnancy (72), local production of melatonin may serve an additional paracrine role. One target may be the placental trophoblast cells, which secrete the "pregnancy hormone" human chorionic gonadotropin (hCG). In two separate laboratories, it was found that high micromolar to millimolar concentrations of melatonin *in vitro* significantly elevated hCG release by human trophoblast cells (40, 71). More recently, the latter group also reported that melatonin at these high levels protects trophoblast cells *in vitro* against hypoxia/reoxygenation–induced inflammation and autophagy (42).

Another potential target for melatonin may be the vascularization of the placenta in early pregnancy through remodeling of the maternal uterine spiral arteries, a process that appears to be defective in preeclampsia—a leading cause of maternal mortality, especially in developing countries. Placental and systemic oxidative stress is considered to be a major underlying mechanism of pathology in preeclampsia (73). With a view toward melatonin's antioxidant properties, it is striking that blood levels and placental synthesis of melatonin decline significantly in women with severe preeclampsia (74–76). In a meta-analysis by Dou et al. (77), these data were corroborated and melatonin levels were found to correlate with the severity of the disease. In terms of a possible beneficial effect of melatonin on placental tissues, Hannan et al. (78) demonstrated that melatonin *in vitro* upregulated antioxidant response genes in human placental trophoblasts as well as in umbilical vein endothelial cells, albeit only at extremely high (1 mM) levels. A recent pilot clinical study by Hobson et al. (79) essentially corroborated these *in vitro* findings. These authors reported modest improvements in the duration of pregnancy in a small cohort of women with preeclampsia who had taken 10 mg oral melatonin three times per day from recruitment until delivery. While it seems highly unlikely that these pharmacological concentrations of melatonin reflect *in vivo* circumstances, these results non-etheless open the door for new therapeutic possibilities to improve clinical outcomes for women with preeclampsia.

MELATONIN AND PARTURITION

Given its proven role as an endocrine signal of night time duration (7, 80) it was not unexpected to find an influence of melatonin on the timing of parturition. Takayama et al. (81) showed that female rats whose endogenous melatonin was eliminated by pinealectomy had no disturbances in estrous cyclicity or in their ability to become pregnant, but they failed to deliver their young exclusively during the daytime (early daytime is the normal birthing phase for nocturnal animals, such as rodents). Instead, the rats gave birth randomly across the 24-h light-dark cycle. However, evening administration of melatonin (i.e., at the time when endogenous levels would normally increase) was effective in restoring the normal daytime birth pattern. Importantly, melatonin was ineffectual when given in the morning or continuously. This strongly points to the ***timing*** of birth in the rat being under circadian control, and that melatonin may serve as a key circadian "gating" signal for this event. These data suggest that the clock may play a subtle, but important role in the reproductive process; however, care must be taken when extrapolating rat data to the human as we are largely diurnal (day-time active), whereas the majority of laboratory rodents are nocturnal (night-time active).

The precise mode of action of melatonin in the mammalian uterus, while still not completely understood, is clearly species-specific. Some earlier reports with rodents (82, 83) showed direct *inhibitory* effects of pharmacological doses of melatonin on uterine contractility as well as the presence of melatonin-specific binding sites in the uterus (84). There are further reports of the inhibitory effects of melatonin on prostaglandin synthesis in various rodent tissues (85, 86). Melatonin has also been shown to modulate calcium signaling in various tissues, including vascular smooth muscle, often via synergistic actions with other receptor-mediated processes (7). Again, care must also be taken when extrapolating data gathered from nocturnal species, such as the laboratory mouse (C57/Bl6), as these and other strains do not produce endogenous melatonin and their parturition physiology is vastly different from that of the human female.

In contrast to the nocturnal rodent, human labor, and delivery are statistically more common during the night phase (87, 88). In view of its nocturnal secretion pattern and the reported effects of melatonin on uterine contractions in other mammals, it seemed reasonable to explore whether melatonin may act as the "temporal gate" in contributing to the contractions that underlie human parturition.

Data from our laboratory (35, 37) have uncovered a significant positive synergistic action of melatonin and oxytocin (OT) on human myometrial smooth muscle cell contractions *in vitro* in which melatonin results in striking amplification of OT-induced IP_3 signaling and OT-induced contractions. These findings may explain the high level of nocturnal uterine contractions found in late term human pregnancy that lead to nocturnal labor (**Figure 1**). More recently, we have also identified a synergistic action of melatonin and OT on myometrial smooth muscle cell induction of the core circadian gene *hBMAL1* (90). BMAL1 is a transcription factor at the core of the circadian system (91, 92) as it serves to regulate the expression of genes whose promoters contain the E-box motif, which includes the melatonin receptors. OT analogs are important tools in obstetrical practice. Continuous infusion of OT agonists is commonly used to induce labor, while OT antagonists are now used to prolong pregnancy in cases of preterm labor. However, prolonged labor induction by application of continuous OT is only effective when high amounts of the hormone are given [due to receptor "desensitization"- (93)]. Unfortunately, continuous OT administration is often accompanied by serious side effects, including fetal distress, uterine rupture, postpartum atony and bleeding. Discovery of a synergism between OT and melatonin signaling (35, 37) could eventually lead to the development of new melatonin + low dose OT medicinal combinations for labor induction without the considerable side-effects of high OT administration. Conversely, studies employing the well-known inhibitory effect of light of circulating melatonin levels have provided corroborating evidence that the nocturnal uterine contractions common to late pregnancy are under melatonin control (94, 95).

A strong, parallel upregulation of the melatonin MTNR1B receptor and oxytocin receptor (OTR) protein in the myometrium of ***laboring*** pregnant, as compared to non-laboring pregnant women has also been demonstrated (35). Parallel trends were noted for *MTNR1A* and *MTNR1B* mRNA expression and for melatonin-binding to these same samples (89) implying melatonin receptor suppression throughout most of gestation with activation (de-suppression) at the end of pregnancy in preparation for parturition. Although uterine quiescence is thought to be a key function of progesterone during pregnancy [e.g., (96, 97)], it is unclear whether melatonin receptor activation in the human myometrium at term pregnancy involves changes in progesterone signaling. Interestingly, in preliminary studies of biopsies from women who entered preterm labor, melatonin receptor protein expression was detected in all samples (98), leading to the fascinating possibility that premature expression of myometrial melatonin receptors may predispose a woman to contractions and preterm labor (99).

MELATONIN IN REPRODUCTIVE AGING

In contrast to early childhood, when high melatonin levels are correlated with low gonadotropin secretion, the presence of low melatonin levels in elderly people appears to be correlated with reproductive aging, i.e., high gonadotropin secretion (100, 101). It is well-established that plasma melatonin levels in elderly individuals are reduced and that the circadian timing of the nocturnal melatonin peak is advanced (102–105).

The normal cessation of female reproductive fertility (menopause) is determined by the inability of the ovaries to produce viable follicles and changes in hormonal secretion that leads to failure of menstrual cycles. Thus, clinically, ovarian aging is characterized by a diminished follicular reserve, which correlates with elevated gonadotropin secretion from the anterior pituitary.

An earlier report of mitigation of depression, and improved mood and sleep quality following melatonin administration to perimenopausal and postmenopausal women (106) could not be confirmed in a study by Amstrup et al. (107) which found no significant effect on quality of life or sleep quality in 81 postmenopausal women who were given pharmacological melatonin nightly for 1 year. However, these authors did report a non-significant trend toward improved sleep quality in a subgroup of melatonin-treated women who had sleep disturbances at initial baseline. Toffol et al. (108) showed that postmenopausal women have lower nighttime serum melatonin levels than perimenopausal women; however, they found no correlations between serum melatonin and FSH or estradiol levels, Beck Depression Inventory score, State-Trait Anxiety Inventory score, BNSQ insomnia score, BNSQ sleepiness score, subjective sleep score, climacteric vasomotor score, or quality of life. The apparent discrepancies in the aforementioned studies is probably reconcilable, since in the Bellipanni and Amstrup investigations pharmacological levels of melatonin (3 mg/night for 6–12 months) were administered, while the Toffol study analyzed physiological and psychological correlations with naturally reduced endogenous melatonin levels. More recently however, long term pharmacological melatonin administration was shown to reduce psychosomatic symptoms in postmenopausal women after 12 months of treatment in a double-blind, placebo study (109). This is consistent with numerous previous studies on the use of pharmacological melatonin in the treatment of sleep disturbances in elderly men and women [cf. (110)].

Some studies have proposed a role for melatonin in ovarian aging, given the supportive and pleiotropic effects of melatonin on ovarian activities, including suppression of oxidative stress, protection of mitochondrial integrity, etc (48, 111, 112). However, as most of the research to date has been in rodents [cf. (113, 114)] a clear etiological relationship between declining endogenous melatonin levels and human menopause has not been adequately demonstrated, nor have sufficiently powered clinical trials with melatonin administration to perimenopausal women been reported.

SUMMARY AND FUTURE PERSPECTIVES

To summarize this short overview of melatonin's association with human female reproduction and fertility, melatonin does not have a strong impact on human puberty, although in this regard its contributions as an endocrine output of the circadian clock need further careful study. Its impact on oogenesis and ovulation, while modest, could still be valuable in the development of new

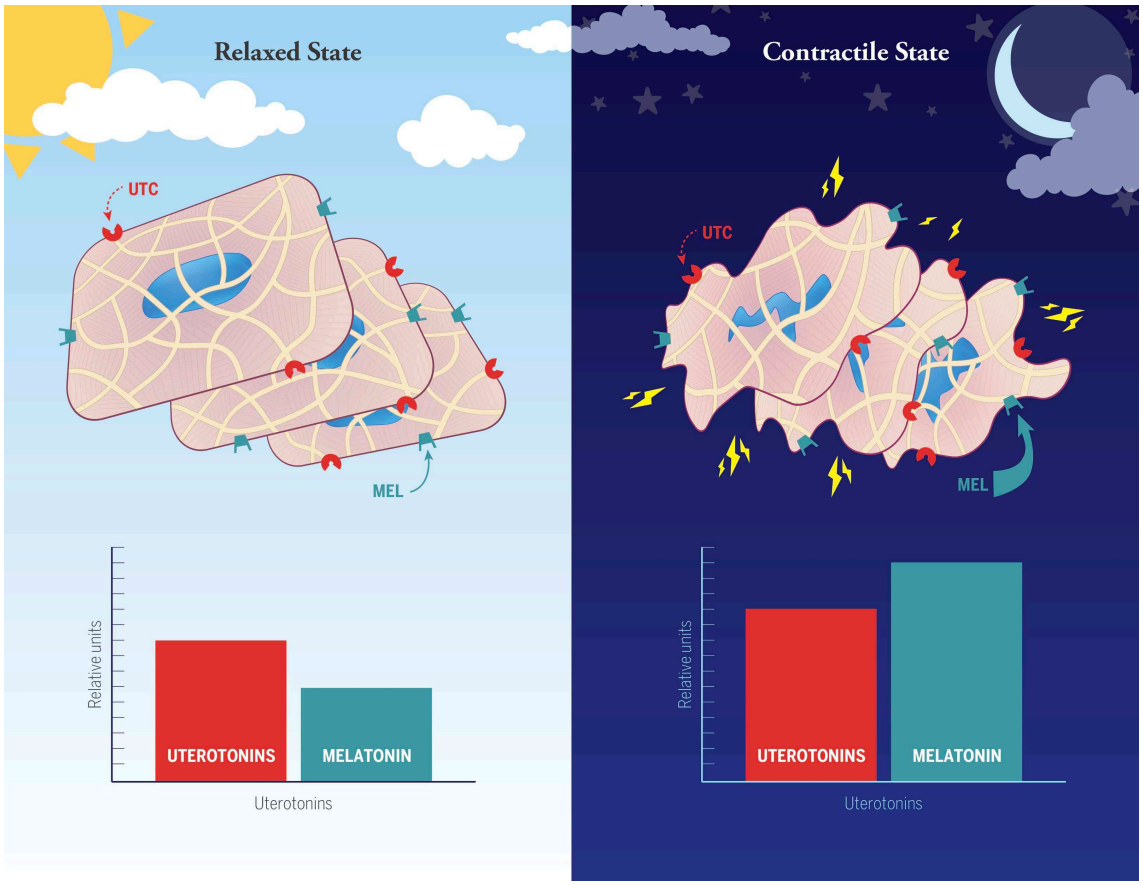

FIGURE 1 | Hypothesized involvement of melatonin with other non-specified uterotonins (e.g., oxytocin, prostaglandins, cytokines) in the establishment of the well-known day night difference in human myometrial contractions during normal late term pregnancy and labor. In this working model the uterine smooth muscle cells are suggested to be in a relaxed state during the daytime because of low levels of melatonin and other unspecified uterotonins (note: the histograms are not meant to be quantitative, but rather to depict relative differences between day and night). High nocturnal uterine contractility is proposed to arise as a synergistic response between uterotonic factors and melatonin, the latter of which is markedly elevated at night. Suppression of nocturnal melatonin levels in late term pregnant women has been demonstrated to significantly reduce uterine contractility (89).

treatments for certain forms of female infertility. Along similar lines, pharmacological melatonin, or analogs may ultimately find application in placental therapeutics, e.g., for treating placental inflammation, oxidative stress, and preeclampsia. And finally, melatonin receptors in the human uterine smooth muscles may offer a surprising new target for the management of labor, both term labor and preterm labor.

There is thus far little substantiated information on the potential association between clinical syndromes involving the reproductive system and melatonin deficits (or excess), or melatonin receptor polymorphisms. Luboshitzky et al. (115) documented increased excretion of the major melatonin metabolite (6-sulfatoxymelatonin) in women with PCOS, although whether this was a consequence of increased or decreased plasma melatonin levels was not apparent. More recently, in a pilot study Tagliaferri et al. (116) administered pharmacological doses of oral melatonin for 6 months to 40 women with PCOS and reported significant improvements in menstrual cyclicity and normalization of androgen balance. A similar reduction of PCOS-related hirsutism and androgen levels after 12 weeks of melatonin supplementation was also found in a recent investigation by Jamilian et al. (117). Thus, the therapeutic use of melatonin in women with PCOS-related conditions shows promise, and should be explored further. Interestingly, Song et al. (118) identified significant gene polymorphism differences in a region of the melatonin type 1 receptor (MTNR1a) between women with polycystic ovarian syndrome (PCOS), but no associated phenotypic differences were seen. Whether other polymorphisms in the human melatonin receptor can be related to other reproductive disorders remains a fascinating though largely uncharted territory.

While research into the potential roles of melatonin in human reproductive physiology continues to expand our intellectual universe after six decades, some common features are apparent. Firstly, melatonin can potentially reach every cell of the body, conveying both circadian information (via plasma melatonin rhythms) and serving as a paracrine modulator of local oxidative

state, inflammatory responses, autophagy, etc (e.g., in the ovary and placenta). Some of these actions are likely to be melatonin receptor-dependent, while others may be receptor-independent. In some cases, melatonin may serve as a permissive or synergistic signal, affecting the response of tissues to other molecules (e.g., oxytocin in the uterus). As an ancient molecule that has taken membership in a wide array of cellular processes in all kingdoms of living organisms over the eons of terrestrial biological evolution, melatonin's involvement in human reproduction is best described to be subtle, diverse, and essential. For example, from *in utero* fetal programming to the timing of parturition, and from influences on metabolism in key reproductive tissues to modulation of neuroendocrine rhythms, melatonin appears to make contributions to all of these processes.

On a closing note, it is critically important to again make the distinction between physiological effects of melatonin and pharmacological consequences of melatonin administration. This warning is of course not unique for melatonin—consider for example another circadian hormone like cortisol. Similarly, when evaluating target effects of melatonin one must remain attentive to species-specific differences in the responsiveness of any tissue to rhythmic and also non-rhythmic levels of melatonin. Developmental stage, gender differences and genetic variabilities all can affect how a reproductive tissue will respond to melatonin. In this regard, it behooves the melatonin researchers of the future to maintain an open mind and an eye for the unexpected. Clearly, melatonin and/or novel analogs of melatonin will eventually find their place in the armamentarium of reproductive medicine in ways that we probably can't even imagine.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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