



# An Examination of the Putative Role of Melatonin in Exosome Biogenesis

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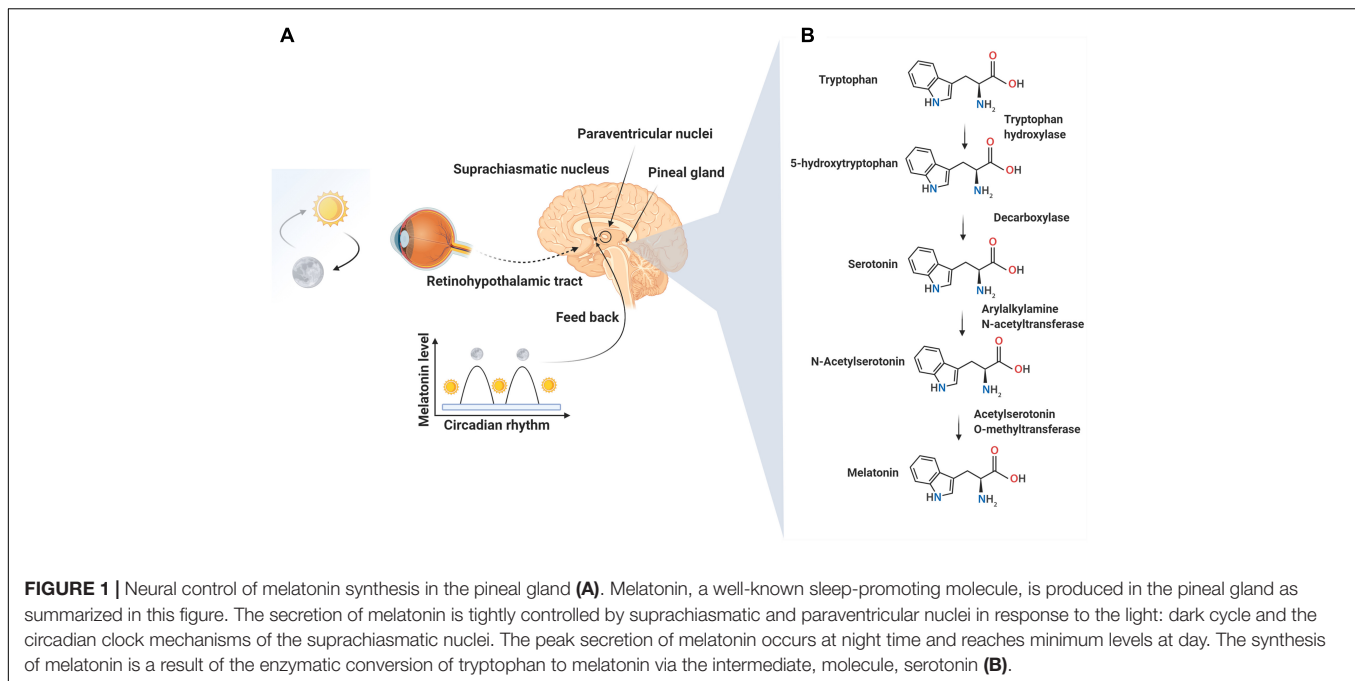
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During the last two decades, melatonin has been found to have pleiotropic effects via different mechanisms on its target cells. Data are abundant for some aspects of the signaling pathways within cells while other casual mechanisms have not been adequately addressed. From an evolutionary perspective, eukaryotic cells are equipped with a set of interrelated endomembrane systems consisting of intracellular organelles and secretory vesicles. Of these, exosomes are touted as cargo-laden secretory vesicles that originate from the endosomal multivesicular machinery which participate in a mutual cross-talk at different cellular interfaces. It has been documented that cells transfer various biomolecules and genetic elements through exosomes to sites remote from the original cell in a paracrine manner. Findings related to the molecular mechanisms between melatonin and exosomal biogenesis and cargo sorting are the subject of the current review. The clarification of the interplay between melatonin and exosome biogenesis and cargo sorting at the molecular level will help to define a cell's secretion capacity. This review precisely addresses the role and potential significance of melatonin in determining the efflux capacity of cells via the exosomal pathway. Certain cells, for example, stem cells actively increase exosome efflux in response to melatonin treatment which accelerates tissue regeneration after transplantation into the injured sites.

**Keywords:** melatonin, exosome biogenesis, interplay signaling pathways, cross talk, paracrine activity

## INTRODUCTION

Numerous bioactivities and therapeutic effects of melatonin have been reported, especially within the last two decades. This hydrophobic molecule is produced by the pineal gland, specifically during the night, and in perhaps the mitochondria of all other cells (Tan et al., 2013; Reiter et al., 2020; **Figure 1**). Melatonin functions via well-described signaling pathways in host cells (Wei et al., 2020). When used by humans, melatonin is typically taken in the evening to improve sleep and, under some clinical conditions, also throughout the day (Castillo et al., 2020; Matar et al., 2021). By means of juxtacrine interactions, cells can transfer soluble biomolecules and factors to other cells at near and remote distances in a paracrine manner using nano-sized vesicles identified as exosomes (Whitham et al., 2018). Exosomes, which range from 40 to 200  $\mu\text{m}$ , develop a suitable



**FIGURE 1 |** Neural control of melatonin synthesis in the pineal gland **(A)**. Melatonin, a well-known sleep-promoting molecule, is produced in the pineal gland as summarized in this figure. The secretion of melatonin is tightly controlled by suprachiasmatic and paraventricular nuclei in response to the light: dark cycle and the circadian clock mechanisms of the suprachiasmatic nuclei. The peak secretion of melatonin occurs at night time and reaches minimum levels at day. The synthesis of melatonin is a result of the enzymatic conversion of tryptophan to melatonin via the intermediate, molecule, serotonin **(B)**.

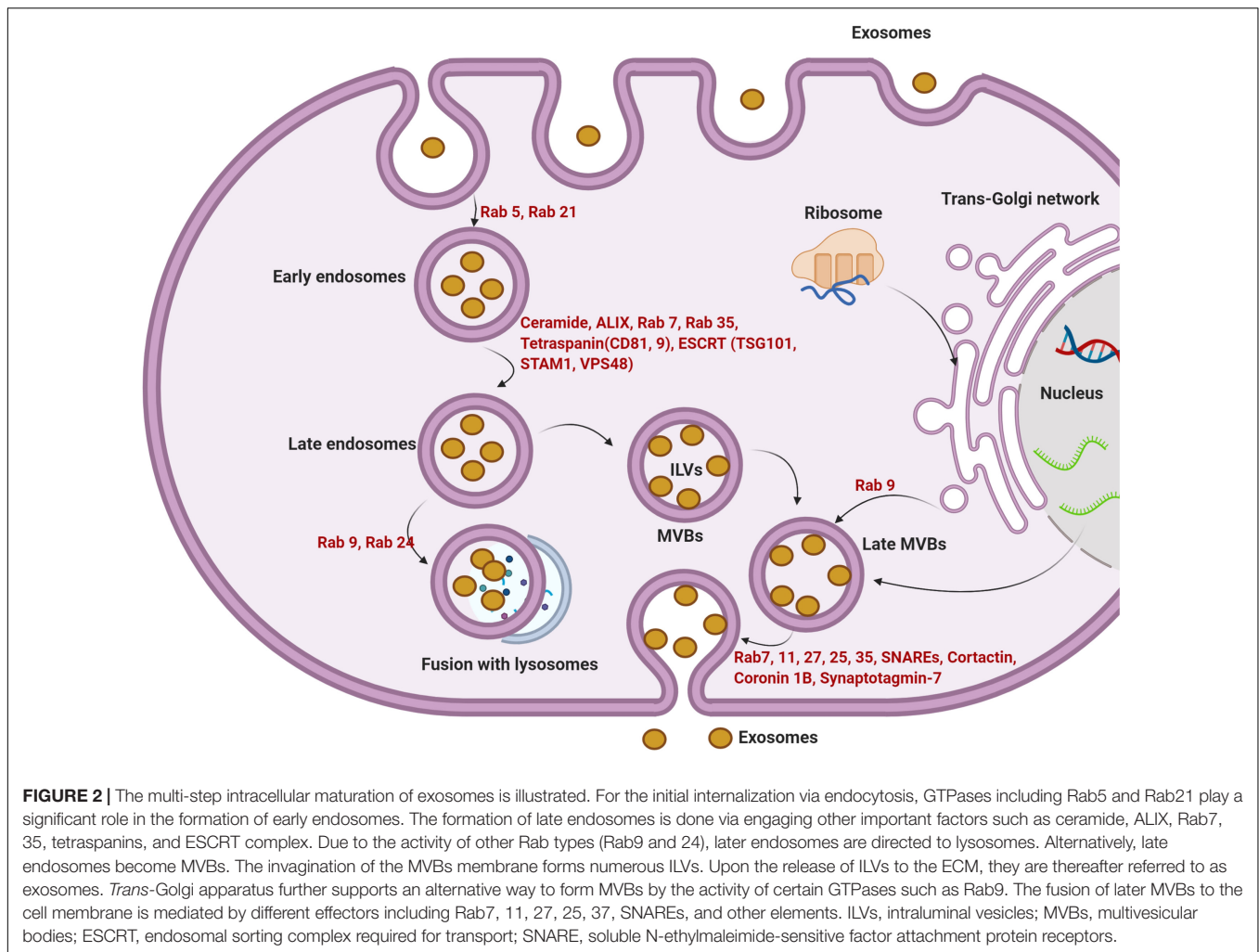
biological platform of biomolecule exchange and mutual crosstalk. Distinct intracellular mechanisms for sorting, trafficking, and abscission have been described, all of which are relevant to exosome biogenesis (Soekmadji et al., 2017). The presence of various genomic and proteomic factors inside exosomes makes them important agents that promote/inhibit certain signaling pathways once they reach their target cells (Minciacchi et al., 2015). Cells release the exosomes under physiological and pathological conditions in response to different factors. Whether the quantity and quality of exosomal cargo differ in different states is the subject of debate (Hooper et al., 2020). Research progress has led to a partial understanding of the intracellular mechanisms involved in exosome biogenesis. Several factors involving the regulation, synthesis, and abscission of exosomes require further studies. Various signaling cascades with multiple effectors are differentially regulated by endogenous or exogenous molecules which in turn influence exosome homeostasis (Bagheri et al., 2018). Within a cell, reciprocal crosstalk between the exosome-contained molecules and other signaling pathways regulate the paracrine capacity via the modulation of exosome biogenesis and abscission (Hassanpour et al., 2018a; Xu et al., 2018). Recent work has focused on the regulatory impact of melatonin on exosome biogenesis (Yoon et al., 2020). This review provides information related to the association of exosomes with melatonin signaling pathways. These pathways have been shown to determine and/or enhance the paracrine actions of melatonin.

## EXOSOME BIOGENESIS AND FUNCTION

Exosomes are small microvesicles of endosomal origin. Exosomes are functionally linked to multivesicular bodies (MVBs) and

released after the fusion of MVBs with the cell membrane (Figure 2; Théry et al., 2009). Exosomes originate from terminal endosomes that are formed by the inward budding of the MVB membrane. In large MVBs, numerous intraluminal vesicles (ILVs) harboring exosomes are obvious (Minciacchi et al., 2015). Of note, ILVs are enriched in various cytosolic components such as soluble proteins, DNA, RNA, or microRNA. The receptor-mediated fusing of MVBs with the cell membrane releases the ILVs, now known as exosomes, into the extracellular matrix (ECM) (Valadi et al., 2007; Sahu et al., 2011). The complex protein machinery consists of four separate proteins including ESCRT-0, -I, -II, and -III and constitute the endosomal sorting complex required for transport (ESCRT) which accounts for MVB formation, vesicle budding, and cytosolic component cargo sorting (Figure 2; Record, 2014). The integrity of ESCRTs and complementary proteins is central to exosome biogenesis (Hurley, 2015). All the ESCRT subunits act via an ubiquitin-dependent pathway. The ubiquitin-binding subunits of ESCRT-0 sequester the ubiquitinated proteins at specialized domains of the endosomal membrane. Following the sequestration of target molecules, ESCRT-I and -II join the complex to develop a high-affinity recognition domain for the ubiquitinated proteins. The addition of the final subunit, ESCRT-III, causes the invagination of the lipid membrane and isolation of ILVs inside the MVB. Vps4 protein provides the energy required to separate buds from the MVB to form ILVs after the addition of ESCRT-III to the ESCRT-0, -I, and -II complexes (Figure 2; Ageta and Tsuchida, 2019). It is postulated that ESCRT machinery may influence exosomal quantity, size, and major cargo protein (Colombo et al., 2013).

The apparent specificity and function of exosomes have been determined. They are known to not only harbor exhausted biomolecules but also may contain specific therapeutic molecules



with the ability to alter the function of target cells in relation to the immune response, antigen presentation, multiple signaling transduction actions in distant cells far from the original host cell (Huang-Doran et al., 2017; Hessvik and Llorente, 2018). The critical roles of exosomes have been implicated in multiple biological phenomena including important functions in tissue growth and development (Yáñez-Mó et al., 2015). However, direct evidence for the exact mechanism of action of exosomes on target cells is lacking. Using an N-ethyl maleimide-sensitive agent, an attachment protein receptor (SNARE) and membrane-bound Rab-GTPase, mainly Rab27, 11, and 35, were identified. Via these elements, MVBs are directed to the plasma membrane for the release of ILVs, exosomes, into the extracellular matrix (ECM) (Figure 2; Rezaie et al., 2018).

Evidence points to the paracrine and autocrine activity of released exosomes (Fitzner et al., 2011; Colombo et al., 2014). How released exosome contents act on specific distant cells in an autocrine manner rather than interacting with neighboring cells requires further investigation. The autocrine activity likely occurs in response to conditions when the host cells require stimulation or to recycle cargo protein (Xu et al., 2018). Numerous studies

have explored the exosome cellular uptake and intracellular trafficking in acceptor cells. Several lines of evidence show that exosome influx is achieved through different pathways including direct fusion with the host cell membrane, receptor/ligand interaction, and internalization. Macropinocytosis, phagocytosis, and endocytosis are non-specific internalization mean for the transfer of exosomes into the cytosol (Mulcahy et al., 2014).

The rate of endocytosis increases when the generation of filopodia and lamellipodia is initiated. The distribution of cell membrane via cytoskeletal remodeling provides a platform to determine their uptake by different pathways (Kamerkar et al., 2017). The existence of CD47 on the exosome surface mediates the “do not eat me” signal and helps them to evade internalization into the target cells (Kamerkar et al., 2017). CD47 also has an essential function to increase exosome transit time in the biofluids and blood (Zheng et al., 2020). Unlike the internalization pathway, the receptor/ligand interaction relies on the specific binding of ICAM-1 at the exosome surface with the LFA-1 receptor on the target cell plasma membrane (Segura et al., 2005). Data suggest the existence of specific molecules on the exosome’s surface that determine exosome

internalization (Huang-Doran et al., 2017; Hessvik and Llorente, 2018). Whether receptor/ligand interaction, fusion, and the internalization pathway are dominant in exosome uptake under certain conditions remain undefined.

## MELATONIN BIOGENESIS AND MECHANISM OF ACTION

N-acetyl-5-methoxytryptamine, known as melatonin, is produced in the pineal gland and likely in the mitochondria of all other tissues; this ubiquitous molecule has highly pleiotropic effects (Claustrat and Leston, 2015). Pineal melatonin secretion into the blood and cerebrospinal fluid is linked to the light/dark cycle (Figure 1; Reiter et al., 2014). Other cells that synthesize melatonin may release the product into the extracellular space but not into the blood. While the pineal gland is touted as the main site of melatonin synthesis, the total amount of melatonin originating from the pineal is small compared to that produced in other cells (Acuña-Castroviejo et al., 2014; Touitou et al., 2017). In the pineal gland, the endogenous rhythm of secretion is regulated by the activity of suprachiasmatic nuclei, the activity of which, in turn, is influenced by the light perception by the retina (Figure 1; Pfeiffer et al., 2018). Significant roles of melatonin include conveying circadian information related to the daily cycle of light and darkness and the regulation of immune system function, antioxidant defenses, glucose metabolism, angiogenesis, etc (Manchester et al., 2015; Tordjman et al., 2017; Zhou et al., 2018). The binding of norepinephrine to adrenergic receptors on the pinealocytes is the accepted mechanism leading to the nocturnal synthesis of melatonin by the pineal gland. Following norepinephrine-adrenergic receptor engagement, the activation of adenylate cyclase increases cAMP and *de novo* synthesis of serotonin-N-acetyl transferase, the rate-limiting enzyme in melatonin production (González et al., 2012). In addition to the ligand-receptor pathway, the melatonin synthesis rate may be influenced by intracellular tryptophan and substrate (serotonin) availability and by folic acid and pyridoxine (Zhao D. et al., 2019). Two membrane-bound melatonin receptors (MT1 and MT2) have been identified on pinealocyte membranes which trigger the changes in cAMP through inhibitory G proteins. The increase of cAMP activates downstream effectors like phospholipase-C and protein kinase-C through the MAPK/PI3K/Akt axis. It has been suggested that melatonin, because of its high lipophilicity, directly crosses the cell membrane to enter the blood and cerebrospinal fluid. This capacity facilitates melatonin's bio-distribution and access to all cells in the organism (Tan et al., 2018).

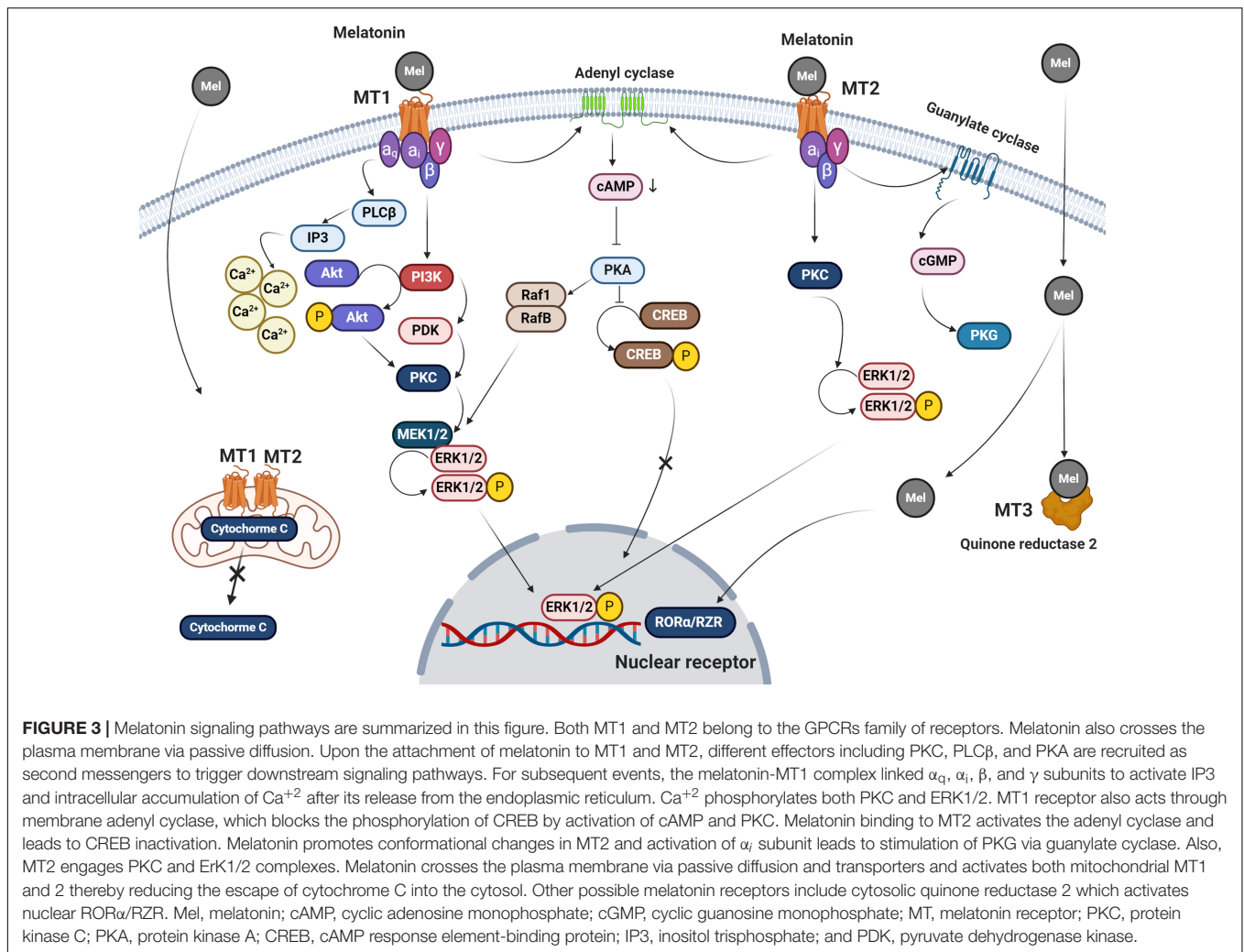
## MELATONIN SIGNALING PATHWAY

The melatonin membrane receptors, MT1 and MT2, belong to G-protein coupled receptor (GPCR) subfamily and are widely distributed, accounting for many of melatonin's pleiotropic functions (Figure 3; Dupré et al., 2018). Besides

its receptor-mediated actions, melatonin also enters cells where it has receptor-independent functions (Hardeland, 2009). Once melatonin crosses the plasma membrane, it may also bind to a cytosolic receptor, MT3. The underlying mechanisms supporting the exact role of intracellular MT3 need further investigation.

MT1 and MT2 have seven transmembrane domains and they have several different signaling pathways within cells (Salon et al., 2011). Studies targeting MTs and GPCR indicate that the binding of melatonin to these receptors leads to increased cAMP production, phosphorylation, calcium movements, and morphological adaptation (Ellisdon and Halls, 2016). Results show that MT1 inhibits forskolin-stimulated cAMP formation, phosphorylation of cAMP-responsive element-binding protein (CREB), and PKA activity after melatonin binding (Figure 3; Emet et al., 2016). The activation of MT1 by melatonin is thought to trigger ERK1/2 responsible for cytoskeleton filament remodeling in neuronal cells (Moreno et al., 2020). MT1 and MT2 have glycosylation sites in the extracellular N-terminal region while the cytoplasmic tail consists of fourth intracellular loops and cysteine residues with palmitoylation capability (Dubocovich and Markowska, 2005). The binding of melatonin to MT2 is in accord with phase-shifting and circadian rhythms while MT1 blunts neural firing and phase-shifting (Hunt et al., 2001; Hardeland, 2009). Both MT1 and MT2 are heterodimers and attached to G proteins like  $\alpha_{12}$ ,  $\alpha_{13}$ ,  $\alpha_1$ , and  $\beta$  and  $\gamma$ . The function of melatonin inside the cells is associated with the activation of diverse subunits of G-proteins connected to MTs and the pattern of downstream interactions.

The activation of MT2 by melatonin is responsible for the reduction of intracellular cAMP ratio and activation of protein kinase C and phospholipase C (Figure 3; Hunt et al., 2001; Rivera-Bermúdez et al., 2004; Birnbaumer, 2007). Activation of G-proteins mediates membrane permeability, allowing ion channels to be opened (Hardeland, 2009). Because of cGMP elevation, it is postulated that this secondary messenger enhances calcium uptake via the cyclic nucleotide-gated channels (Rimler et al., 2007). Melatonin is involved in several transcription processes and gene expression via the regulation of CREB and ERK (Cecon et al., 2018). Binding to multiple intracellular effectors is an additional mechanism-specific to melatonin which is associated with its lipophilic properties (Emet et al., 2016). This is based on the results showing the existence of several putative cytosolic melatonin receptors including enzyme quinone reductase 2 (the MT3 receptor), ROR $\alpha$ /RZR nuclear receptors, and calmodulin (Figure 3; Jockers et al., 2008). Evidence points to immune cells as the main sites of ROR $\alpha$ 1 and ROR $\alpha$ 2 responsible for mediating melatonin effects while RZR $\beta$  is abundant in the pineal gland (Pandi-Perumal et al., 2006). ROR $\alpha$  was shown to be an active receptor in the regulation of antioxidant enzymes (Hardeland, 2009). The function of ROR $\alpha$  in rat cardiomyocytes after myocardial ischemic reperfusion injury shows its basic role in the control of inflammation and oxidative stress (Figure 3; He et al., 2016). It is similarly observed that melatonin reaches different cellular constituents through its lipophilic property and via receptor-independent pathways (Venegas et al., 2012).



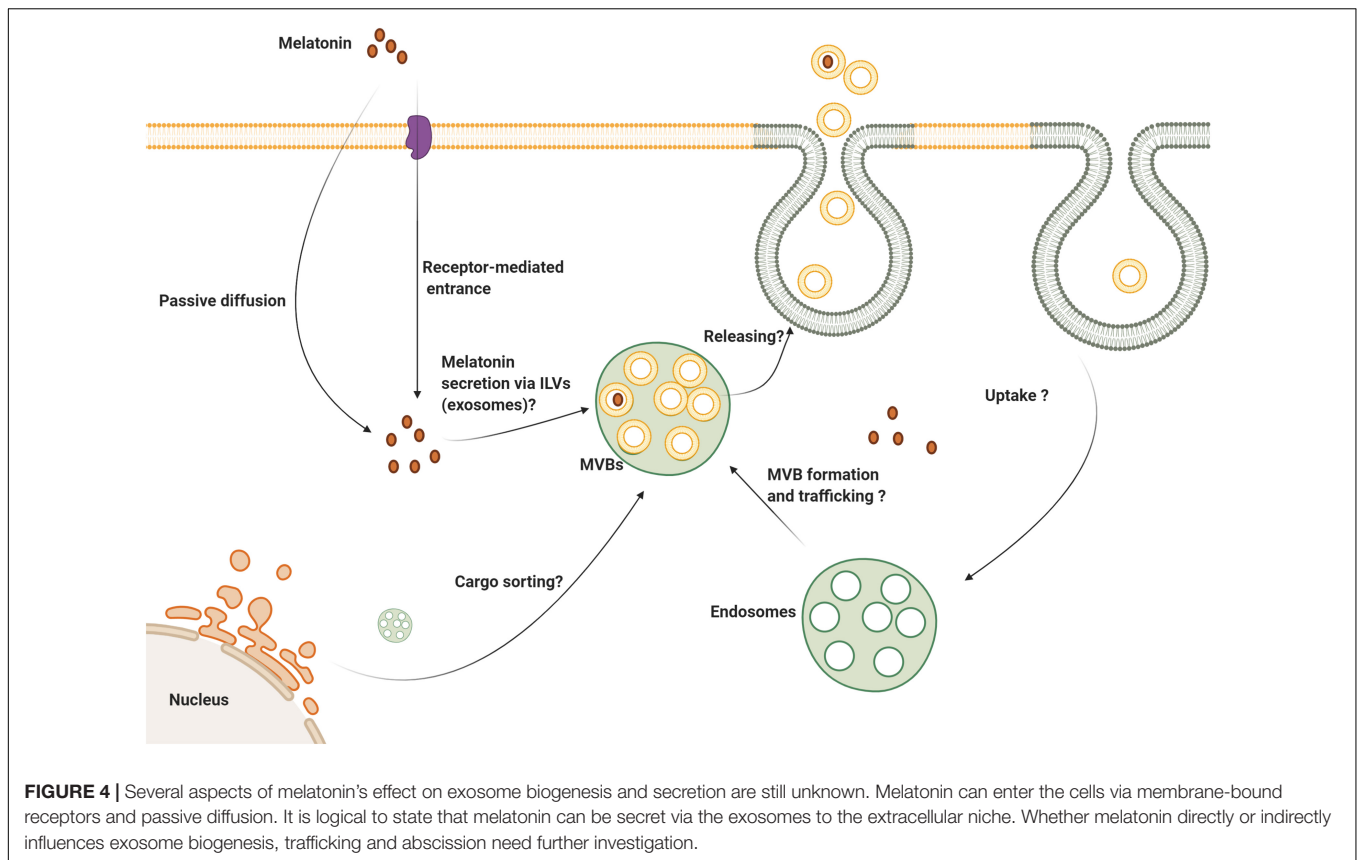
## EFFECT OF MELATONIN ON EXOSOME BIOGENESIS AND RELEASE

Due to the unique diverse functions of melatonin, it is considered a possible modulator of exosome biogenesis and function (Yoon et al., 2020). How melatonin modulates the function of exosomes under physiological and pathological conditions is under investigation (Figure 4). By identifying the mechanism of action of melatonin as it relates to exosome function, will allow researchers and clinicians to take advantage of the known functions of these important microvesicles. As an example, this may allow for the control of stem cell therapeutic effects in the context of regeneration and other pathological conditions. Using a variety of experimental models in stem cell research, knowledge related to the possible role of exosomes in the alleviation of pathological changes has accumulated (Bang and Kim, 2019; Park et al., 2019; Zhao L. et al., 2019). Recent investigations have established the synergistic effect of melatonin and adipose-derived mesenchymal stem cell (MSC) exosomes in suppressing inflammation, oxidative stress, and apoptosis in a rat model

of hepatic ischemia/reperfusion (I/R) (Sun et al., 2017; Chang et al., 2019).

These data are also consistent with other observations showing that exosomes of bone marrow MSCs pre-conditioned with melatonin have benefits in MSCs via paracrine which improved therapeutic efficacy on I/R induced acute renal failure (Alzahrani, 2019; Zahran et al., 2020). However, the exact relevance of melatonin as to its activities, such as those conveyed by exosomes, remains unknown (He et al., 2016). Based on our recent findings melatonin alters exosome size and production in bovine granulosa cells in a dose-dependent manner in which higher melatonin concentrations contribute to the elevated release of small-sized exosomes (Pournaghi et al., 2021). This may be due to the promotion of ILVs formation and/or alterations of exosome physicochemical properties.

Whether melatonin is also transferred via exosomes between the cells needs further investigation. The direct passive diffusion of melatonin through cell membranes is accepted. Also, oligopeptide and glucose transporters are alternate routes to deliver melatonin into the cytosol (Venegas et al., 2012; Mayo et al., 2017). Given its lipophilicity and rapid passive diffusion,



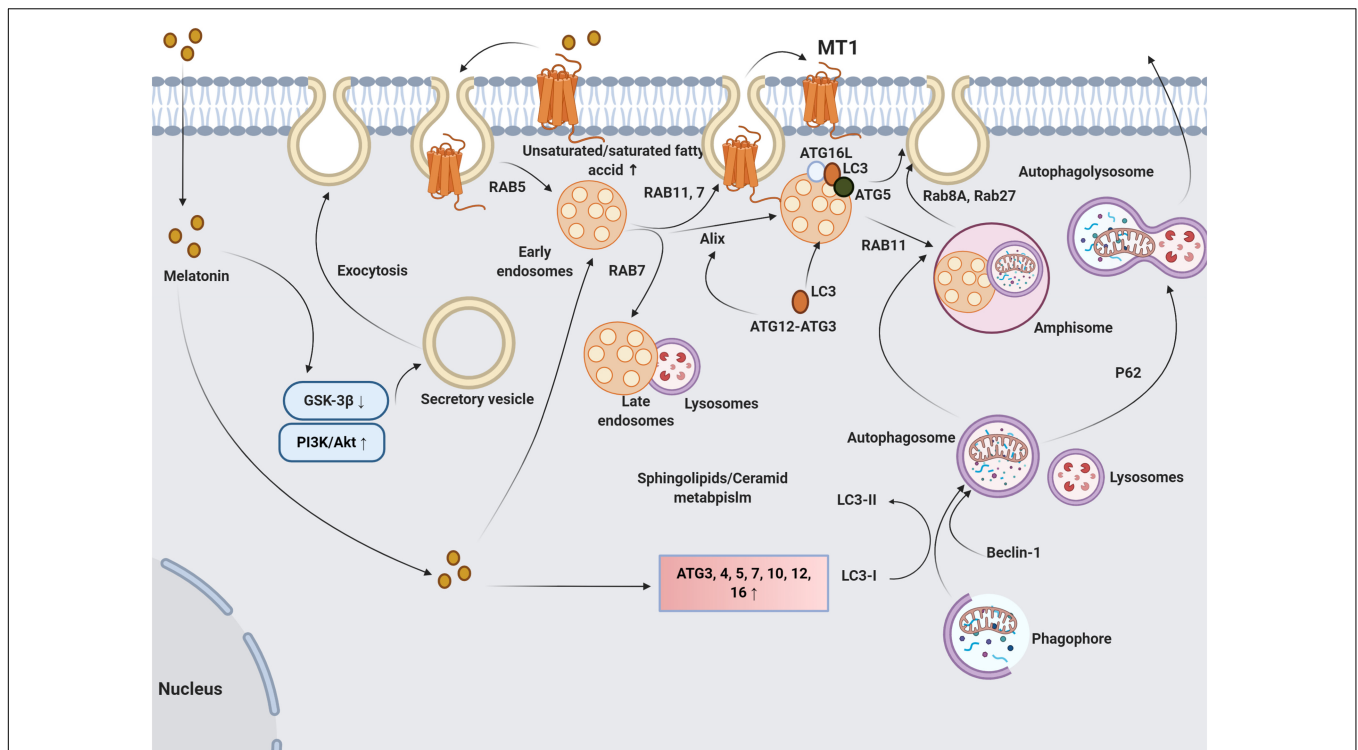
**FIGURE 4** | Several aspects of melatonin's effect on exosome biogenesis and secretion are still unknown. Melatonin can enter the cells via membrane-bound receptors and passive diffusion. It is logical to state that melatonin can be secret via the exosomes to the extracellular niche. Whether melatonin directly or indirectly influences exosome biogenesis, trafficking and abscission need further investigation.

it is speculated that melatonin transfers horizontally between cells independent of membrane transporters. Interestingly, the transfer of MT1 via internalization, endocytic transport has been explored directly (Abd-Elhafeez et al., 2017). Once melatonin binds MT1, vacuolar sorting machinery transfers the internalized MT1 to the early endosomes using Rab5. Due to the activity of other GTPases like Rab11 and 7, endosomes carrying MT1 can be recycled to the plasma membrane. Alternatively, late endosomes with surface Rab7 activity fuse with the lysosomes (Abd-Elhafeez et al., 2017). The inhibition of  $\beta$ -arrestin-2 by Valproic acid reduces basal endocytic transport of MT1 (Abd-Elhafeez et al., 2017). These data show the potential capacity of exosome transport in intracellular localization of the downstream effectors of the MT signaling pathway (**Figure 5**).

Although investigated to a lesser extent, the modulation of exosome biogenesis and changes in cargo composition in host cells has been studied. Using an *in vitro* model of Alzheimer's disease, Ozansoy et al. (2020) showed a reduction of tau carried by exosomes in human neuroblastoma cell line SHSY-5Y incubated with melatonin. They also observed that melatonin pre-treatment of cells before amyloid- $\beta$  incubation did not affect exosomal tau levels or the intracellular hyperphosphorylated tau content (Ozansoy et al., 2020). A recent histological analysis of sheep testes showed enhanced exosome secretion from cytoplasmic extensions of telocytes located in seminal vesicles after administration of melatonin (Abd-Elhafeez et al., 2017).

Following the application of melatonin, the morphology of telocytes was changed and the number and diameter of releasable exosomes increased (Abd-Elhafeez et al., 2017). This issue contrasts with some other results and the difference regarding melatonin's effect on exosome secretion may be associated with the final concentration of melatonin (either physiological or pharmacological dose). On the basis of the published data, and despite its unique functions in different cell types, it is proposed that melatonin has the potential to regulate exocytosis and exosome delivery.

It is unclear why and how melatonin increases or decreases exosome secretion under different metabolic circumstances. Whether the dose of melatonin or the physiological state of a cell is influential in the exocytosis rate should be further examined. Moreover, the precise signaling pathways involved in exosome biogenesis and secretion upon treatment with melatonin remain unclear. Attempts to identify a correlation between melatonin receptors and exocytosis yielded evidence of relevant signaling cascades (Liu et al., 2015). This group found that the Akt/GSK-3 $\beta$ /CRMP-2 axis is an intermediate molecular process between MT2 and exocytosis in rat hippocampal neurons (**Figure 5**; Liu et al., 2015). Upon treatment of neurons with melatonin and activation of MT2, the PI3K/Akt axis suppressed GSK-3 $\beta$  activity and reduced phosphorylation of CRMP-2 allowing axonogenesis, exocytosis, and synaptic transmission (Liu et al., 2015). This pathway may be relevant to the regulation of exosome secretion and other extracellular vesicle types.



**FIGURE 5 |** The multiple possible means of molecular cross-talk between melatonin and exosome synthesis machinery are summarized here. Irrespective of how melatonin enters cells, it activates PI3K/Akt complex and inhibits GSK-3 $\beta$ , leading to fusion of secretory vesicles with plasma membrane and exocytosis. However, the underlying mechanisms have not been fully established. Changes in plasma membrane fluidity by an alteration of fatty acid composition (unsaturated/saturated fatty acid ratio) facilitate the exosome release. Also, melatonin promotes specific GTPases including Rab7 and 11 which accelerate the fusion of late MVBs with the plasma membrane. Upon binding of melatonin to MT1, the melatonin-MT1 complex is transferred to the early endosomes via vacuolar sorting machinery such as Rab5. The activation of Rab7 and 11 promotes endosomal secretion and recycles MT1 to the plasma membrane. Further activation of Rab7 directs endosomes with MT1 toward lysosomes which regulate the innate cell response to melatonin. How Rab 7 determines the fate of endosomes with melatonin (secretion or enzymatic digestion) needs further explorations. The interplay between melatonin and autophagy signaling pathway also influences the activity of exosome molecular machinery. Melatonin stimulates autophagic response directly via the activation of ATG4, 5, 7, 10, 12, and 16 and increases the LC3II/I ratio. These features promote the formation of autophagolysosomes and autophagic exocytosis. Melatonin enhances the autophagic machinery including LC3, ATG3, 5, 12, and 16L on the late endosome membrane that facilitates the fusion of these elements with the plasma membrane. Melatonin also increases the intersection of autophagic vacuoles and exosomes into compartments named amphisomes which have multiple fates. Amphisomes can fuse with lysosomes via the activation of P62. The activity of Rab8 and 27a initiates the autophagic release of amphisomes to the ECM. GSK-3, glycogen synthase kinase 3; ATG, autophagy-related protein; P62, sequestosome 1; LC3, microtubule-associated protein 1A/1B-light chain 3; and PI3K/Akt, Phosphatidylinositol-3-kinase/Protein kinase B.

A quantitative analysis of multiples genes related to different signaling transduction pathways in *Saccharomyces cerevisiae* under oxidative stress revealed mild to moderate expression of 29 genes regulating transmembrane transport activity after treatment with melatonin (Sunyer-Figueres et al., 2020). It may be that whether and how simultaneous activation of MT1 and 2 influence exosome delivery is determined by the physiological state of the host cell. In addition to the molecular pathways that participate in exosome release, other findings indicate changes in the physicochemical properties of the cell membrane as a result of melatonin exposure (Sunyer-Figueres et al., 2020). The report claimed a moderate rise in the synthesis of unsaturated fatty acids and sphingolipids upon treatment with melatonin, indicating an increase in membrane flexibility and fluidity (Sunyer-Figueres et al., 2020). In our previous work, we found that melatonin can alter the unsaturated/saturated fatty acid ratio by increasing Arachidonic acid, Oleic acid, and Linoleic acid levels and change

the flexibility of the cell membrane, leading to enhanced exosome delivery (Pournaghi et al., 2021).

This contrasts with the application of long-chain saturated fatty acids like palmitic acid on exosome release (Maly and Hofmann, 2020). It was suggested that the addition of 25  $\mu$ M palmitic acid suppressed exosome release from prostatic carcinoma PC3 cells (Maly and Hofmann, 2020). The selective inhibition of sphingolipid-metabolizing enzymes such as neutral sphingomyelinase reduced exosome secretion from neural cell lineages (Yuyama et al., 2012). Collectively, the data suggest that an alteration in the metabolism of fatty acids by melatonin may be another molecular mechanism that influences exosome release from host cells.

It is also apparent that melatonin regulates exocytosis which is involved in the progression of apoptosis and autophagy (Luo et al., 2020). Based on the results of several different experiments, certain intracellular and cell-membrane bound

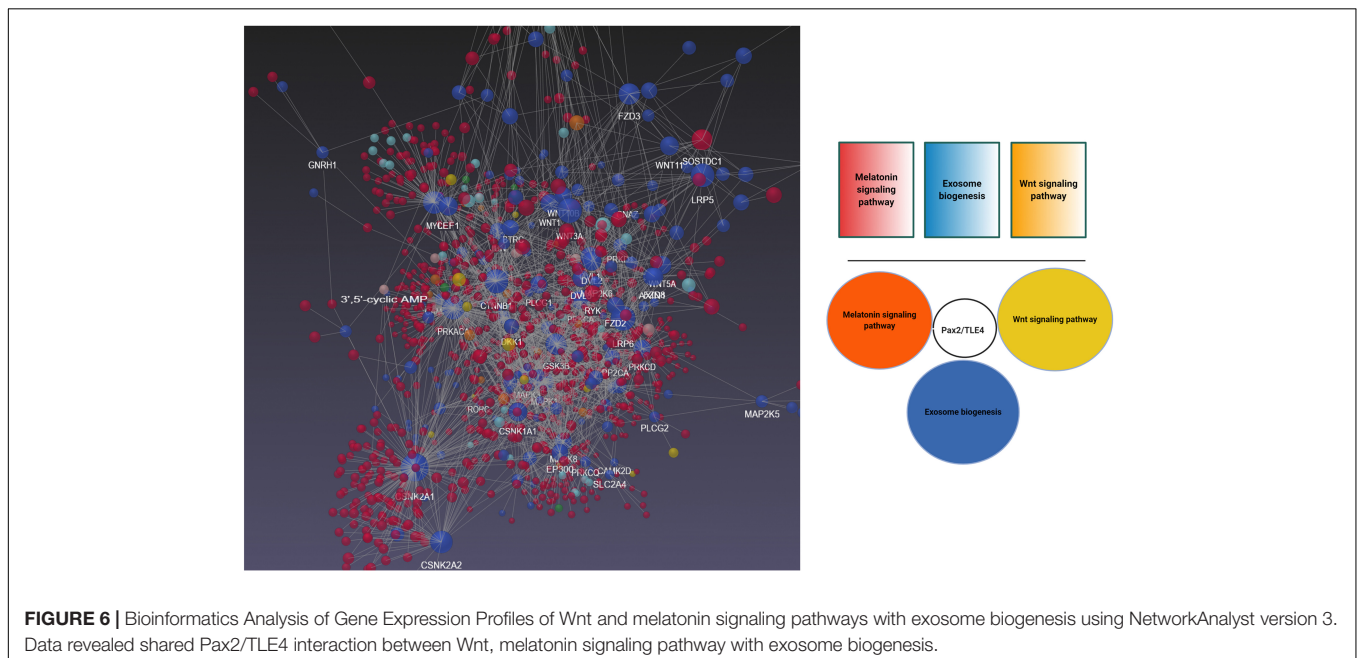
proteins are responsible for the fusion and transfer of multiple sets of vesicles. The emerging data point to the existence of subsets of molecular mechanisms shared between autophagy and exosome abscission (Xu et al., 2018). Autophagy-related proteins (ATG), consisting of different subsets, are key regulators of the autophagic response in the cytosol and plasma membrane (Hassanpour et al., 2018a). Data show that ATG5 and ATG16L1 are shared between autophagy signaling and exosome biogenesis (Guo et al., 2017). ATG5 separates vacuolar proton pumps, namely  $V_1V_0$ -ATPase, and inhibits acidification inside the MVBs, allowing the fusion of MVBs with the plasma membrane and release of ILVs (Figure 5; Xu et al., 2018). The treatment of endothelial progenitor cells with melatonin after exposure to advanced glycation end product decreased cell toxicity by autophagic response mediated by the up-regulation of P-62 and LC3II/I ratio (Jin et al., 2018). It is also suggested once the conjugation of LC3 is initiated via the activity of the ATG3-ATG12 complex, Alix, which belongs to the ESCRT complex, is provoked and interacts with LC3 (Murrow et al., 2015). Thus, cells with LC3 activity achieve the autophagic response and exosome abscission by the interaction of Alix with LC3, showing the integration of autophagy mechanism and exosome biogenesis. In the support of this claim, the inhibition of LC3 conjugation interrupts both exosome secretion and the autophagic response (Murrow et al., 2015). It should be noted, however, that the promotion of autophagy does not necessarily accelerate the secretion of ILVs.

It has been reported that the activation of autophagy-related machinery by rapamycin increased MVB-autophagosome fusion and generated hybrid vacuoles, referred to as amphisomes (Xu et al., 2018). Amphisomes release exosomal and autophagy contents using specific GTPases including Rab8a and Rab27a. Under some circumstances, amphisomes fuse with the cell

membrane and are redirected toward lysosomes leading to the formation of autophagolysosomes and a reduction of exosome delivery (Figure 5; Villarroja-Beltri et al., 2016). It was suggested that the expression of GTPases such as Rab27a and Rab27b was increased in cumulus cells after treatment with melatonin for 7 days (Pournaghi et al., 2021).

Studies using endothelial progenitor cells treated with diabetic sera showed reduced exosome secretory capacity (Hassanpour et al., 2018b). Considering the shared machinery regulating exosome abscission and autophagy, the promotion of the autophagic response would be expected to stimulate exosome secretion in the presence of melatonin. However, direct evidence for autophagy-mediated exosome release after melatonin treatment is lacking.

To better understand the possible modulatory effect of melatonin on exosome biogenesis, we performed bioinformatic analyses using NetworkAnalyst version 3, and molecular networks were generated by the application of Network Visual Analytics (Figure 6). Based on our previous experience, the Wnt signaling pathway affects exosome biogenesis and secretion during physiological and pathological conditions of the endothelial lineage (Bagheri et al., 2018). To mine the relationship between melatonin and exosomes, we performed a bioinformatic analysis based on common genes related to the melatonin signaling pathway, exosomes biogenesis, and the Wnt cascade. Our analyses showed that transducin-like enhancer of split 4 (TLE4) is a common effector that cross-links these three pathways. TLE4 belongs to the TLE family of transcription co-repressors. The TLE family members do not directly attach to the DNA. These proteins could generate repressor complexes after binding to Runx2, Hes1, and T cell factor/lymphoid enhancer-binding factor (TCF/LEF) (Zhang et al., 2019). Upon the activation of toll-like receptors (TLRs)





**TABLE 1** | Melatonin could alter exosomal cargo.

Type of study	Study design	Outcome	References
<i>In vivo</i> and <i>in vitro</i>	• Injection of bone marrow MSC-derived exosomes exposed to melatonin in a rat model of diabetic wound healing	IL-1 $\beta$ ↓, TNF- $\alpha$ ↓, and IL-10↑, Arg-1↓ and iNOS↓, Improved angiogenesis rate (CD31 and $\alpha$ -SMA↑), collagen synthesis↑, Increased M2 to M1 polarization via activation of PTEN/AKT signaling pathway	Liu et al., 2020
<i>In vivo</i>	• Hepatoprotection of adipocyte-derived exosomes in mice received melatonin compared to mice fed with a high-fat diet and resistin	Fatty acid accumulation in the liver↓, endoplasmic reticulum stress↓, hepatic steatosis↓, resistin-induced AMPK $\alpha$ phosphorylation↓, increased resistin mRNA degradation↑, Bmal1 transcriptional inhibition↑, and m <sup>6</sup> A RNA demethylation in adipocytes ↑	Rong et al., 2019
<i>In vitro</i> and <i>in vivo</i>	• Melatonin-treated hepatic carcinoma-exosomes effect on peritoneal macrophages immunosuppression capacity	Peritoneal macrophage phosphorylated STAT3↓, IL-6↓, IL-1 $\beta$ ↓, IL-10↓, PD-L1↓, and TNF- $\alpha$ ↓ in THP-1 cells,	Cheng et al., 2017
<i>In vivo</i>	• Maternal intraperitoneal of melatonin on lipopolysaccharide-induced neonatal brain inflammation	Microglial activity↓, Endoplasmic reticulum↓, Autophagy efflux↑, eIF2 $\alpha$ phosphorylation↑, P62 and LC3↑, SIRT1↑, miR-34a↓, miR146a↓, and miR-126↓, Inflammatory response↓	Carloni et al., 2016
<i>In vivo</i>	• Injection of MSCs exosomes preconditioned with melatonin in a rat model of renal I/R injury	Tubular epithelial cell necrosis↓, Immune cell infiltration, and inflammation↓, BUN and creatinine levels ↓, Renal BUN and creatinine levels↑, MDA↓, NOx↓, HIF-1 $\alpha$ ↑, HO1↑, Apoptosis-related markers↓, Angiogenesis↑	Alzahrani, 2019

↓ Decrease; ↑ Increase; IL-1 $\beta$ , interleukin 1 beta; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; ARG1, arginase; iNOS, inducible nitric oxide synthase;  $\alpha$ SMA, smooth muscle alpha-actin; CD31, platelet endothelial cell adhesion molecule; AMPK $\alpha$ , AMP-activated protein kinase  $\alpha$  subunit; P62, sequestosome-1; LC3, microtubule-associated protein 1A/1B-light chain 3; PD-L1, programmed death-ligand 1; STAT3, signal transducer and activator of transcription 3; eIF2, eukaryotic initiation factor 2; BUN, blood urea nitrogen; MDA, malondialdehyde; NOx, nitrogen oxides; HIF-1 $\alpha$ , hypoxia-inducible factor 1 alpha; HO-1, heme oxygenase-1.

including TLR2, 3, 4, and 7 by different ligands, the expression of TLE3 and 4 was initiated under pro-inflammatory status (Zhang et al., 2019). Based on our analyses, we found an interaction of TLE4 with Pax2 shared between melatonin and exosome biogenesis. Previously, the close interaction of melatonin and Wnt signaling pathways has been documented. As a result, the activation of Wnt4 through the ERK1/2-Pax2-Egr1 axis in preosteoblasts after exposure to melatonin increased bone formation capacity (Li et al., 2019). Whether activation of Pax2/TLE4 complex modulates the biogenesis of exosomes needs further investigations.

Research advances have confirmed the role of melatonin as a key player in the regulation of cell paracrine responses via exosome biogenesis. Further investigations should be directed at the characterization of the signaling pathways that regulate exosome biogenesis after treatment with melatonin. Identifying the molecular events by which melatonin modulates exosome biogenesis will likely soon become an active area of investigation.

## EFFECT OF MELATONIN ON EXOSOME CARGO SORTING

Within the last decade, *in vivo* and *in vitro* evidence has shown that melatonin changes the molecular composition of exosome cargo (Table 1; Ozansoy et al., 2020). Progressive chronic changes such as chronic kidney disease, profoundly decrease the innate restorative capacity of MSCs (Zhao et al., 2020). Moreover, molecular analyses confirmed the existence of several factors, in the particular cellular prion protein (PrPC) and miR-4516, which participate in the regulation of angiogenesis (Alzahrani, 2019). These changes occurred concurrently with other desirable effects

such as anti-aging actions and improvement of mitochondrial function after transplantation of MSCs treated with melatonin. These studies revealed that melatonin stimulates MSCs to release exosomes that are highly enriched with miR-4516-PrPC (Yoon et al., 2020). Other pro-angiogenic and anti-inflammatory factors have also been identified in MSC-derived exosomes preconditioned with melatonin (Liu et al., 2020). Exosomes isolated from melatonin-treated MSCs accelerated healing via an increase of microvascular density and suppression of inflammation in the rat model of diabetic wound healing (Liu et al., 2020).

Evidence for therapeutic effects of exosomes in hepatic disease has also been noted (Shen et al., 2014; Colica and Abenavoli, 2018). A recent study explored the hepatoprotection of adipocyte-derived exosomes in mice who received melatonin (Rong et al., 2019). Compared to mice fed with resistin and a high-fat diet, melatonin application produced adipocyte-derived exosomes which are capable of reducing hepatic steatosis and endoplasmic reticulum stress (Rong et al., 2019). These data suggest that melatonin changes the exosome cargo and decreases phosphorylation of AMPK- $\alpha$  in Thr172 residue and that this activity prohibits endoplasmic reticulum and the development of hepatic steatosis (Rong et al., 2019). Owing to melatonin's pleiotropic effects, one scenario is that melatonin displays a regulatory mechanism on small molecules known as miRNAs which are subsequently released by exosomes (Hardeland, 2018a). Melatonin appears to alter m<sup>6</sup> A RNA demethylation in adipocytes and changes the expression of resistin (Rong et al., 2019).

A large number of cancer cell exosomes, known also as oncosomes, containing multiple sets of miRNAs are involved in tumor metastasis and expansion (Cufaro et al., 2019; Jaiswal and Sedger, 2019). Melatonin is known to regulate

the transcription of certain miRNAs such as miR-155 and miR-21 in cancer cells. These miRNAs are responsible for tumor growth and metastasis (Hardeland, 2018b; Hunsaker et al., 2019). The alteration of exosomal miRNAs by melatonin not only inhibits the horizontal transfer of anaplastic-associated bio-information by exosomes but also recruits immune cells to the cancer site (Alfonsi et al., 2018; Wang et al., 2019). Studies have shown a moderate reduction in pro-inflammatory cytokines including TNF- $\alpha$ , IL-6, -10, -1 $\beta$  and programmed death-ligand 1 by macrophages incubated with hepatocellular carcinoma-derived exosomes pre-conditioned with melatonin (Cheng et al., 2017). The regulation of STAT3 may play a critical role in the immunomodulatory actions of carcinoma-derived exosomes (Cheng et al., 2017). Under other circumstances, such as lipopolysaccharide-induced Alzheimer's disease in rat neonates, melatonin decreases the pathological complications via the autophagy-SIRT-1 pathway and down-regulation of multiple miRNAs including miR-34a, -146a, and -126 (Meng et al., 2014; Carloni et al., 2016). Similarly, Heo et al. (2020) showed that melatonin-stimulated exosomes isolated from adipose-derived mesenchymal stem cells suppressed inflammation. These exosomes were enriched with multiple miRNAs such as miR-34a, -124, and -135b compared to non-treated melatonin exosomes.

Since various accessory factors are shared by autophagy and exosome signaling pathways, it is presumed that melatonin inhibits the progression of Alzheimer's disease by activation of exocytosis via both autophagy efflux and exosome secretion. This possibility as it relates to neurodegenerative diseases needs further analysis.

A recent study reported by Wang et al. (2020) injected blood-derived exosomes of melatonin-treated rats into the brain after focal cerebral ischemia. Melatonin changed the exosomal miRNAs profile and reduced ischemic changes via the promotion of angiogenesis, neurogenesis, and suppression of apoptosis. It is felt that melatonin alters cargo sorting in the exosomes. Attempts to investigate the effect of melatonin on exosome functions have not yielded a definition of the molecular mechanisms by which it controls exosome dynamics.

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## CONCLUDING REMARKS AND PERSPECTIVES

Research progress has led to the partial clarification of melatonin's efficacy in the modulation of exosomal biogenesis. What has been uncovered is that melatonin can alter the exosome biogenesis, intracellular trafficking, and abscission at the molecular levels by engaging different effectors. Further investigations should be focused on the identification of commonly shared pathways between exosome biogenesis and the melatonin signaling cascade. Based on the dose- and context-dependent activity of melatonin on the target cell populations, the findings related to these interactions will have to be interpreted with caution. Questions related to the heterogeneity and functions of effectors shared between two signaling pathways are still unanswered. Advancements in molecular biological analyses and improved technologies will be aids in clarifying melatonin-exosome interactions at different subcellular levels.

## AUTHOR CONTRIBUTIONS

HA, AR, MHe, MHa, SH, and ES collected the data and prepared the draft. SG performed the bioinformatic analysis. RR and RJR edited and supervised the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare 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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