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MINI REVIEW

Melatonin, endocrine pancreas and diabetes

Abstract: Melatonin influences insulin secretion both in vivo and in vitro. (i) The effects are MT_1-and MT_2-receptor-mediated. (ii) They are specific, high-affinity, pertussis-toxin-sensitive, G_i-protein-coupled, leading to inhibition of the cAMP-pathway and increase of insulin release. Furthermore, melatonin inhibits the cGMP-pathway, possibly mediated by MT_2 receptors. In this way, melatonin likely inhibits insulin release. A third system, the IP_3-pathway, is mediated by G_q-proteins, phospholipase C and IP_3, which mobilize Ca^{2+} from intracellular stores, with a resultant increase in insulin. (iii) Insulin secretion in vivo, as well as from isolated islets, exhibits a circadian rhythm. This rhythm, which is apparently generated within the islets, is influenced by melatonin, which induces a phase shift in insulin secretion. (iv) Observation of the circadian expression of clock genes in the pancreas could possibly be an indication of the generation of circadian rhythms in the pancreatic islets themselves. (v) Melatonin influences diabetes and associated metabolic disturbances. The diabetogens, alloxan and streptozotocin, lead to selective destruction of β-cells through their accumulation in these cells, where they induce the generation of ROS. β-cells are very susceptible to oxidative stress because they possess only low-antioxidative capacity. Results suggest that melatonin in pharmacological doses provides protection against ROS. (vi) Finally, melatonin levels in plasma, as well as the arylalkylamine-*N*-acetyltransferase (AANAT) activity, are lower in diabetic than in nondiabetic rats and humans. In contrast, in the pineal gland, the AANAT mRNA is increased and the insulin receptor mRNA is decreased, which indicates a close interrelationship between insulin and melatonin.

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Key words: circadian rhythm, diabetes, insulin, melatonin, melatonin receptors, pancreatic β-cell, radicals

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Divergent results characterize the history of melatonin–insulin relationships

The Romanian group of C.I. Parhon [1, 2] was the first to perform and report systematic research on the importance of the pineal gland in connection with carbohydrate metabolism. In the following years, many publications opened a discussion concerning the importance of the pineal for glucose metabolism, which is still controversial today [3–14]. The reason why these early results were ambiguous probably lies in the use and application of whole pineal extracts; melatonin was not isolated from the bovine pineal by Lerner until 1958 [15], and its molecular structure was not identified until 1959 [16]. In the following review, an attempt will be made to outline the often rather complex and controversial discussion of the influence of the pineal gland and melatonin on the pancreatic islet, as well as on insulin secretion and glucose metabolism. Moreover, a possible link between melatonin and the efforts regarding diabetes prevention will be examined. Within this context, the early results of Parhon and colleagues concerning the pineal impact on the β-cell, on insulin secretion and on glucose metabolism were of particular importance. Their results can be summarized as follows: a pineal peptide, designated 'pinealin' by the authors, was described as being similar to insulin in that it displays hypoglycemic, anabolic, anti-cholisterinemic and glomerulotrophic characteristics [17]. Pinealin increases glucose tolerance and enhances muscular and hepatic glycogenesis after a glucose challenge. In contrast, pinealectomy decreases insulin secretion, glucose tolerance, as well as muscular and hepatic glycogenesis and increases blood pyruvate concentration [3–14]. The subsequent, frequently cited publications of the Spanish group led by E. Blázquez [18–21] are in accordance with the earlier results, which indicated that pinealoprive hypoglycemia is linked to further 'paradiabetic' metabolic disturbances in pinealectomized animals. By pinealectomy or bilateral sympathetic denervation of the pineal gland [22–24], significantly reduced insulin levels, increased blood glucose [25], and disturbances in glucose tolerance were observed. These effects could be counteracted totally or partially by melatonin application [26]. Within this context, observations are important which indicate that melatonin suppresses the onset of type 1 diabetes, whereas pinealectomy has the contrary effect [27, 28]; it was also reported that insulin increases plasma melatonin concentrations [29],

as well as the melatonin content of the pineal gland [30]. These listed publications question earlier, but also contemporary results, which substantiate the observation of hyperglycemia because of the application of pineal extracts [31–33]. It cannot be ruled out that ambivalent results within the Romanian group itself are of pivotal importance in this context, since the same authors reported in 1971 [13] that pinealectomy of rats reduced insulin secretion after 48 hr of fasting. The glucose-stimulated insulin secretion, in contrast, was found to be increased. Hormone fractions of the pineal gland were initially observed to lead to an increase, but later to a decrease in insulin secretion [34]. Subsequently published work with similar results implied that melatonin reduces the glucose-induced liberation of insulin in rat and mice [35], but not basal insulin secretion, and that chronic infusion of melatonin had very little effect on the decrease of insulin and none at all on blood glucose levels [36]. Furthermore, it has been published that high melatonin levels, because of blinding [37], or of exogenous melatonin application, raise blood glucose levels [38–43], whereas blood glucose levels decrease [44, 45] and insulin level increases [12, 46–49] because of pinealectomy. Most of the above-mentioned authors conclude that the pineal gland has a suppressive effect on the activity of the β-cell, because melatonin lowers the insulin levels in rat [50–52] and these effects are coincident with a reduction in glucose tolerance [53, 54]. Based on these results and the realization that an increased insulin level exerts an inhibitory effect on the pineal gland and melatonin [55, 56], a functional antagonism between insulin and melatonin has to be assumed. This fact is even more striking taking into account the reduced insulin levels during the night in man, which are coincident with elevated nocturnal melatonin concentrations; contrary to the situation of low-level melatonin during the day [57] together with high levels of insulin. In addition, the diabetic patient is largely devoid of any circadian melatonin rhythm [58, 59]. Although these early publications were characterized by contradictory results concerning a putative enhancing or inhibiting effect of melatonin on insulin, they were in accord insofar as a close functional link between melatonin and several characteristic features of carbohydrate metabolism was determined.

There are, however, publications which deny or question any influence of melatonin on the pancreatic β-cell and insulin secretion. Within this context, an early publication should be mentioned which stated that no inhibitory influence of melatonin on the glucose-induced insulin secretion could be confirmed in Syrian hamsters [60]. Furthermore, neither in mice [61], nor rats [62, 63] could the influence of melatonin on basal or glucose-stimulated insulin secretion be detected. Some reports on avian species are ambivalent in this respect as well. Whereas John et al. [64] stated in 1983 that melatonin did not lead to an alteration of the blood glucose levels in turkeys, in either the photo- or the scotophase, a later result from 1990, on pigeons, confirmed the blood glucose-increasing effect of melatonin after application [40]. Particular attention should be given to those publications that take the age of the animals under examination or the duration of the photoperiod into consideration. For example, melatonin caused hyperglycemia in newborn pigeons, whereas, in the adult bird, hypoglycemia was detected [65]. In budgerigars, after melatonin application under natural light conditions, as well as under long-day conditions, hyperglycemia was observed in contrast to short-day conditions, which caused hypoglycemia [66, 67]. In addition to the duration of the photoperiod, the divergent amounts of melatonin given or the time of day should significantly influence the effect of the pineal hormone on the glucose metabolism in birds. Thus, melatonin regulation of glucose homeostasis in birds depends on age and species. The cited findings clearly indicate that melatonin plays a pivotal role in glucose homeostasis in birds [65], modulated by norepinephrine and epinephrine [68]. On the other hand, there is no question that factors uneffected by environmental light modify the sympathetic nerve tone and can also influence pineal function [29, 69]. In connection with the importance of melatonin for the regulation of insulin secretion and blood glucose, several reports have been published that examined the impact of melatonin on the growth hormone, on the insulin-like growth factor (IGF-1) and on body weight. Again, some publications confirm the positive influence of melatonin on the growth hormone and on body growth [40, 70–72], while others deny such a connection [73]. One focus of attention is the inhibitory effect of melatonin on the hypoglycemia-induced increase of the growth hormone and body weight [51, 74–78]. In this context, results have been presented showing that melatonin decreases the food consumption in a dose-dependent manner [79] and that pinealectomy or sympathetic denervation of the epiphysis (caused by bilateral extirpation of the upper cervical sympathetic chain of the sympathetic trunk) increases food consumption and weight gain [80]. Concerning the importance of melatonin on IGF-1, no conclusive results have been reached. Some publications favor the idea of a positive effect of melatonin on IGF-1 [70, 72, 81], which is reduced [72] after pinealectomy, but some publications report a negative role of melatonin on IGF-1 [82–85]. Although a systematic evaluation of the above-mentioned results is not possible, general agreement exists that melatonin exerts a relevant impact on glucose metabolism. In connection with seasonal influences of the pineal organ on nutritional and metabolic interrelations in the male Wistar rat and with integrated endocrine and hypothalamic regulatory mechanisms, please refer to the review article of D. Peschke [86].

Recent results characterize a decrease of insulin secretion after application of melatonin

In retrospect, those publications obviously appear more convincing (because of their superior experimental design and inclusion of chronobiological aspects) that report an inhibitory effect of melatonin on insulin secretion. High levels of insulin were always measured when melatonin levels were reduced (during the day); on the other hand, low levels of insulin in parallel with high glucose levels were always measured during the night [87]. Similar results

were detected for humans, when glucose levels were maintained at a constant level by permanent glucose infusion, thus avoiding post-prandial effects [57]. In accordance with these results are studies in rat which proved that, with increasing age, the synthesis of melatonin declines, whereas the synthesis of insulin and leptin increases [50] and that melatonin is able to stop the age-related insulin increase [52]. Complementary to these results are publications [55, 56, 58] reporting that melatonin levels are reduced in diabetic hamsters. On the other hand, there is evidence for a diabetes-preventing effect of melatonin, whereas pinealectomy increases the risk [27, 28]. Further data likewise demonstrate that melatonin influences both glucose metabolism and insulin secretion from the β-cell. Removal of the pineal gland, however, cannot be compensated for by mimicking melatonin concentrations in plasma only [88]. By using an efficient dynamic perifusion system, the influence of indolamines on insulin secretion could be examined. This study was conceived to investigate the function of melatonin and serotonin under the direct control of insulin secretion from the pancreatic islets [89]. Explanted rat islets were treated with melatonin or serotonin, either in a repetitive or long-term manner, in the perfusion system. Repetitive administration of melatonin and serotonin alone did not alter the basal insulin secretion from the explanted islets even at a pharmacological level. However, the insulin response to a specific (glucose) or nonspecific (KCl) stimulus was significantly reduced while the islets were treated with melatonin. This effect was reversible and repeatable. On the other hand, serotonin significantly enhanced both glucose- and KCl-stimulated insulin release. These data show that melatonin and serotonin have a direct effect on insulin secretion from the pancreatic islets; results were reproduced in the following years [90, 91] and confirmed by others [92, 93]. To elucidate the previously discussed putative interaction between melatonin and insulin, glucose, insulin, and melatonin levels of type 2 diabetic patients as well as type 2 diabetic Goto Kakizaki (GK) rats were analyzed by radioimmunoassay. Expression of pancreatic melatonin receptors, pineal insulin receptors and arylalkylamine-*N*-acetyltransferase (AANAT) was determined by real-time RT-PCR. The AANAT enzyme activity was measured in pineal homogenates. Diabetic patients showed a decrease in melatonin levels, while in the pancreas of GK rats an upregulation of the melatonin receptor mRNA was determined. The pancreatic islets of GK rats showed expression of the mRNA for the pancreatic melatonin receptor (MT_1), which had previously been identified in rats and in INS1 cells [91]. Besides their presence in animal cells, the MT_1-receptor transcript was also detected in human pancreas by RT-PCR. Whereas the rat pancreatic mRNA expression of the MT_1-receptor was significantly increased, the activity of the pineal AANAT enzyme was reduced. The latter observation was in parallel with (lower) plasma melatonin levels. The insulin receptor mRNA of the pineal gland was found to be reduced in GK rats. These observations suggest a functional interrelationship between melatonin and insulin, and may indicate a reduction of melatonin in the genesis of diabetes [94].

Melatonin receptors of the pancreatic β-cell

After having found convincing proof for the influence of melatonin on the pancreatic β-cell and a certain functional interrelationship between melatonin and insulin, a second line of query remained open: Are these effects specific and are pancreatic β-cells endowed with melatonin receptors, as detected by Reppert and co-workers [95–97] in other cell types? To clarify this question, the following points and strategies were pursued. Because it is generally accepted that melatonin exerts some of its biological effects through specific, high-affinity, pertussis-toxin-sensitive, G_i protein-coupled receptors, the putative melatonin receptor of pancreatic islets was blocked using the melatonin antagonist luzindole and the specific binding of [^{125}I] iodomelatonin to the membrane was inhibited by the addition of the nonhydrolyzable guanosine triphosphate analogue guanosine 5′-O-(3-thiotriphosphate) (GTPγS). Both GTPγS and luzindole caused near normalization of the melatonin-induced inhibition of forskolin-stimulated insulin secretion [98, 99]. All in all, the results of the luzindole experiments are comparable to the GTPγS findings [90, 91].

To localize putative melatonin receptors within the pancreatic islets autoradiographic studies were additionally carried out. These investigations showed specific binding of 2-[^{125}I]iodomelatonin. In addition, gray level analysis showed that unlabeled melatonin was able to reduce the binding of 2-[^{125}I]iodomelatonin in a dose-dependent manner. Concentrations of unlabeled melatonin of 10^{-9} mol/L produced a 50% reduction in specific binding, whereas concentrations of 10^{-6} mol/L displaced the binding completely.

Likewise, the results of molecular investigations showed that the rat pancreas contains a melatonin receptor, as RT-PCR experiments, using specific primers for the rat melatonin receptor MT_1, showed that mRNA for this melatonin receptor type is expressed in pancreatic tissue. In summary, evidence was collected that the MT_1 receptor is located on the pancreatic islets.

Finally, using molecular techniques, it was demonstrated that a melatonin receptor mRNA identical to that cloned from the rat brain is expressed in pancreas tissue of newborn rats [95]. The specificity of the single amplification product was confirmed by restriction analysis and nested PCR, indicating that it corresponds to the predicted 329-mer MT_1 product. A possible co-expression of the MT_2 receptor [96] in the pancreatic tissue was originally excluded. In summary, the results indicated that a melatonin receptor, most likely the MT_1 receptor, was located in the pancreatic islets of neonate rats and that the pancreatic islets are targets for receptor-mediated melatonin influences [90, 92, 93, 100–102]. As convincing as these molecular results concerning the detection of a melatonin receptor may have been, they had the drawback of being collected on whole pancreatic tissue only. It was therefore crucial to institute a cell system that allowed detection at the level of a single β-cell. Until this was done, no information was available on whether the MT_1 receptors were located on the β-cells or whether the consecutive functional reactions were based on the direct influence of melatonin on β-cells. To examine these aspects, a glucose-responsive,

insulin-producing insulinoma cell line INS1, isolated from rats, was used. Comparable to the results of islets, the competitive receptor antagonist luzindole diminished the insulin-decreasing effect of melatonin. In addition, PCR experiments, using specific primers for the rat melatonin receptor MT_1 showed that this melatonin receptor mRNA is also expressed in the INS1 cells [91, 92]. Evidence was exclusively found for expression of the MT_1 receptor in the pancreatic β-cell model INS1, in the pancreatic islet and in the whole rat pancreas. In contrast, phase-shifting effects on the insulin rhythm in isolated islets of rats after application of melatonin [103] indicated expression of a putative MT_2 receptor on the pancreatic β-cell. By using the recently developed technique of fluorescence-dye-coupled real-time RT-PCR and the primers adapted to this technique, rat pancreatic tissue, isolated islets and INS1 cells were examined for melatonin receptor transcript expression. Details of the procedures for RNA extraction, DNase treatment of RNA and reverse transcription have recently been published [104]. However, a more sensitive DNA-intercalating fluorescence dye was employed throughout PCR experiments in the above-mentioned study. The primer sets were targeted at the exon-2-derived part of the MT_1 and MT_2 transcripts. By using PCR conditions as previously described [104] experiments succeeded in amplifying MT_1 as well as MT_2 mRNA-derived PCR products which were verified by gel electrophoresis and restriction analysis. A quantitative comparison of MT_1 versus MT_2 receptor expression for islet-derived transcripts from batches of islets by real-time RT-PCR indicated that the MT_2 transcript concentration is much lower (86-fold) in this tissue compared to the MT_1 mRNA level. This low-level expression likely explains the lack of conclusive results for the existence of the MT_2 receptor in earlier studies [90, 91]. The function of the pancreatic MT_2 receptor with its own signal transduction pathways within the context of insulin secretion and circadian synchronization will have to be elucidated [105]. Recently, molecular and immunocytochemical investigations established the presence of the melatonin membrane receptors MT_1 and MT_2 in human pancreatic tissue (surgical material) and, notably, also in the islets of Langerhans [105]. Results of a calculation model to determine mRNA expression ratios, as well as subjective analysis of immunoreactions, showed elevated MT_1 receptor expression in comparison to MT_2 expression. The mRNA transcript levels of melatonin receptors appeared to be significantly higher in type 2 diabetic patients than in a control group. An upregulation of receptor expression in type 2 diabetic patients was also observed in immunocytochemical investigations [106]. In addition, the transcription factors RORα, RZRβ, RORγ, and RevErbα were detected in human pancreatic tissue and islets. In correlation with membrane melatonin receptors, data indicate increased mRNA expression levels of RORα, RZRβ, and RORγ in type 2 diabetic patients. Thus, the data demonstrate the existence of the melatonin membrane receptors MT_1 and MT_2, as well as mRNA expression of nuclear orphan receptors in human pancreatic tissue, with upregulated expression levels in type 2 diabetic patients [106]. These data on nuclear melatonin receptors are still preliminary, but complement the results on the better characterized membrane receptors MT_1 and MT_2 without claiming to imply connections to specific functions for insulin secretion within the islet.

Melatonin-receptor-mediated pathways in the β-cell

It is commonly accepted that melatonin exerts some of its biological effects through specific, high-affinity, pertussis-toxin-sensitive, G_i protein-coupled receptors [95–97]. The following signal transduction was mediated by adenylyl cyclase (AC) and, subsequently, by the second messenger cAMP [107–109]. Recent investigations have shown that melatonin reduces the forskolin-stimulated insulin secretion from isolated pancreatic islets of neonate rats, as well as the cAMP production of both pancreatic islets and INS1 cells [90–92, 102, 110]. The adenylyl cyclase activator forskolin was used to stimulate cAMP and insulin levels. As shown earlier, the competitive melatonin-receptor antagonist luzindole completely reversed the cAMP- and insulin-diminishing effects of melatonin [90]. In confirmation of these findings, the $G_i\alpha$-protein-inhibitor pertussis toxin (PTX) abolished the effect of melatonin on the levels of cAMP and insulin as well. Taken together, these results clearly indicate an inhibitory influence of melatonin on the cAMP-signaling pathway of pancreatic β-cells, mediated via $G_i\alpha$-protein-coupled MT_1 receptors. However, the intracellular signaling of melatonin in pancreatic β-cells is not limited to the cAMP-signaling pathway. Recently, it was discovered that melatonin likewise inhibits the guanylatcyclase/cyclic guanosine monophosphate (GC/cGMP) pathway (I. Stumpf, unpublished data). Furthermore, there is evidence for the involvement of the IP_3 system in the signaling cascade of melatonin in a growing number of cell types. In contrast to the uniform cAMP-diminishing effect of melatonin, both IP_3-increasing [111, 112] as well as IP_3-decreasing [113–115] effects of melatonin have been described in different cell types. Previous results, obtained by IP_3-mass assay on extracts from INS1 cell-culture batches, indicated a dose-dependent stimulation of IP_3 release by melatonin [116]. Similar to the cAMP-diminishing effect of melatonin, the competitive melatonin-receptor antagonist luzindole was able to completely abolish the IP_3-liberating effects of melatonin, giving strong evidence for the involvement of melatonin receptors [116]. The type of MT_1 receptor coupled G-protein-subunit stimulating phospholipase C (PLC) can only be hypothesized. In vitro expressed melatonin receptors exhibit differential abilities to stimulate PLC via $G_q\alpha$-proteins [117, 118], and the MT_1 receptor has been seen to couple with $G_q\alpha$-proteins in an agonist-dependent and guanine-nucleotide-sensitive manner in HEK293 cells [119]. However, in NIH 3T3 cells expressing the human MT_1 receptor, melatonin markedly elevates the IP_3-liberating effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), presumably via the $G_{\beta\gamma}$-subunit [107]. It is a well-established fact that carbachol stimulates insulin secretion in pancreatic β-cells by activation of muscarinic acetylcholine receptors, PLCβ, IP_3 release and, ultimately, the elevation of intracellular Ca^{2+} [120–123]. Both melatonin and carbachol showed stimulatory effects on IP_3 release of INS1 cells. Co-stimulation resulted in even higher IP_3

levels [116]. Since IP_3 liberation is stimulated and cAMP release is inhibited by melatonin, which would normally be conflicting signals for insulin secretion, the overall effect on insulin secretion was of interest. These results indicate a predominance of the cAMP- and, subsequently, the insulin-inhibiting pathway for melatonin. These findings confirm earlier results in INS1 cells [90–92] and whole islets [90, 103, 124]. Even the carbachol-induced insulin release that is not cAMP-based showed a reduced peak when melatonin was in the medium. There is no satisfactory explanation for this phenomenon at the moment. After selective inhibition of the cAMP-signaling pathway via PTX, there was a stimulatory effect of melatonin on carbachol- and even forskolin-stimulated insulin release, probably because of activation of the IP_3-signaling pathway by melatonin. As shown in earlier fluorescence-imaging studies, melatonin-induced IP_3 liberation was able to mobilize intracellular Ca^{2+} concentrations [116], a mechanism that is commonly accepted as a trigger for insulin release. PTX proved to be a valuable tool to distinguish between the $G_i\alpha$-dependent cAMP pathway and the $G_i\alpha$-independent IP_3 pathway. The successful inhibition of $G_i\alpha$-proteins was validated in each superfusion that included PTX. The inhibitory influence of melatonin on the cAMP-signaling pathway required a longer incubation period [90, 91]. In contrast, the stimulatory influence of melatonin on IP_3 levels is an instant effect [116]. Comparable effects of pineal hormone fractions have been described: first an increase followed by a decrease in insulin levels [34]. In conclusion, it was found that the melatonin receptors on pancreatic β-cells are coupled to three parallel signaling pathways, with different influences on insulin secretion. In terms of insulin release, there is a predominance of the cAMP-pathway leading to inhibition of insulin secretion. Possibly mediated by the MT_2 receptor, it was recently detected that melatonin likewise inhibits the guanylatcyclase/cyclic guanosine monophosphate (GC/cGMP) pathway and consecutively inhibits the insulin secretion. Melatonin-mediated IP_3 release may play a role in the short-term support of other IP_3-releasing agents, like acetylcholine, or may be related to the activation of protein kinase C (PKC) or the long-term regulation of β-cell functions with enhancing effects on insulin secretion. Thus, the influence of melatonin on pancreatic β-cells and on insulin secretion is connected with a complex pattern of intracellular signal transduction pathways, which includes cAMP-, cGMP-, and IP_3-signaling pathways. Putative signaling pathways of the pancreatic β-cell influenced by melatonin via MT_1- and MT_2-membrane receptors are shown in Fig. 1.

Insulin secretion of the pancreatic β-cell is organized by a circadian rhythm

Various investigators have postulated oscillations of insulin secretion [125] within a range of seconds [126] to periods of between 9 and 14 min under both in vivo and in vitro conditions [127]; furthermore, in clonal pancreatic β-cells with periods of 5 to 8 min, a rhythm was superimposed with 15 to 20 min interval fluctuations [128]. The current opinion is that they are generated by a pacemaker located within the pancreas. These observations were made on decentralized islets of dogs [129], mice [130], rats [131] and humans [125]. Thus, in man, a circadian rhythm of enhanced insulin secretion during the day and a decrease during the night has been described [57]. In this case, plasma-insulin and plasma-melatonin concentrations change in an opposing manner during the 24 hr period, i.e., melatonin peaked when insulin was at a low level and vice versa. In rats, a circadian rhythm determined the nuclear size of pancreatic β-cells, with a peak at noon [132]. Further information on the circadian rhythms of insulin secretion from isolated rat pancreatic islets, maintained in an in vitro perifusion system, was published in 1998 [103]. A circadian pattern has been observed, with periods between 21.8 and 26.2 hr. Adding melatonin as a zeitgeber during analysis of the phase responses in insulin secretion resulted in circadian phase shifts. After melatonin application, the circadian period was maintained, but the amplitude was enhanced. From this experiment it was concluded that an endogenous oscillator is located within the pancreatic islets of the rat that regulates the insulin secretion of the β-cell in a circadian manner. Additionally, important investigations in rat insulinoma cells INS1 have shown that an overnight pretreatment with melatonin resulted in a marked increase in insulin secretion, cAMP-response element-mediated gene expression and insulin-promoter-driven luciferase gene expression in response to glucagon-like peptide 1 (GLP-1) or forskolin [92]. However, prolonged exposure of INS1 cells to melatonin application (12 hr) resulted in sensitization of cAMP-mediated responses to forskolin and GLP-1. This phenomenon may represent the first evidence of a specific physiological role for melatonin-induced sensitization of the pancreatic β-cell, with respect to cAMP signaling [92]. On the other hand, an inappropriate time schedule for the administration of melatonin could induce supraphysiological concentrations of melatonin, resulting in a desensitization of melatonin receptors. Lengthy exposure to melatonin could mimic 'artificial darkness,' causing physiological disturbances, e.g., to glucose metabolism [133], whereas pinealectomy, leading to a lack of melatonin, decreased insulin sensitivity, as well as GLUT4 gene expression [134].

Circadian rhythms of clock genes in the pancreas islets

In order to elucidate the basis for the described pancreatic circadian insulin oscillations, changes in the expression of the following clock genes were investigated on the transcriptional level: *Clock*, *Per1*, *Per2*, *Cry1*, *Tim*, and *Bmal1* during a 24-hr period by real-time RT-PCR. In addition, circadian transcript changes of the clock-controlled output genes *Dbp* and *Rev-erbα* were monitored [135, 136]. The findings suggest the function of a circadian pacemaker in the rat pancreas. Furthermore, evidence of a circadian oscillator in the islets was shown. The presence of PER1 in rat and murine pancreatic tissues as a ~45 kDa protein indicates a translation product of low molecular size. This protein is of the same molecular size as PER1 identified in liver and brain [137]. In INS1 cells, PER1 was detected as a major band in serum-treated cells at approximately 45 kDa, together with a weak band at approximately 60 kDa; both

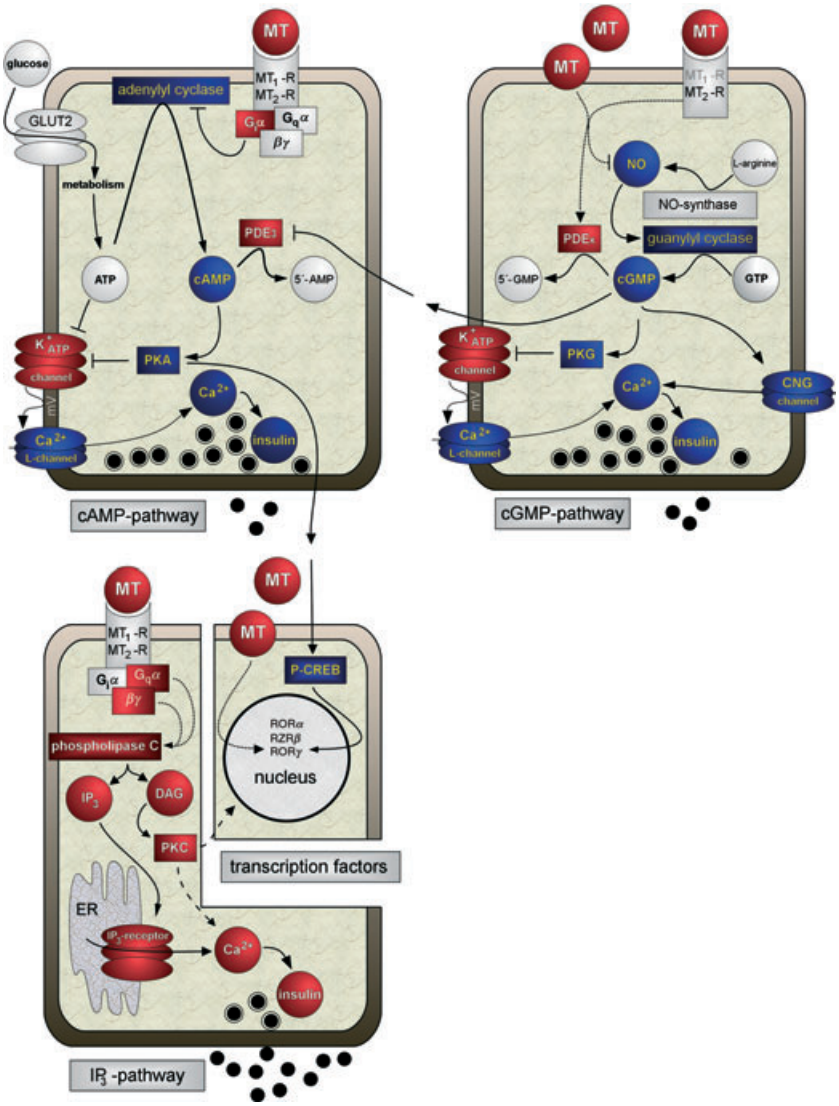

Fig. 1. Putative signaling pathways of the pancreatic β-cell influenced by melatonin via MT_1- and MT_2-membrane receptors considering nuclear orphan transcription factors: (i) the adenylyl cyclase/cAMP pathway mediated via inhibitory G_i-proteins with an insulin-secretion-inhibiting effect, (ii) the phospholipase C/IP_3 pathway mediated via G_q proteins with an insulin-secretion-enhancing effect, and (iii) the cGMP pathway possibly via MT_2-receptors, analogous to the cAMP pathway, with insulin-secretion-inhibiting effect. ATP, adenosine triphosphate; Ca^{2+} L-channel, voltage-dependent Ca^{2+} channel; AMP, cyclic adenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; CNG-channel, cyclic nucleotide-gated channel; CREB, cAMP-responsive element-binding protein; DAG, diacylglycerol; ER, endoplasmic reticulum; $G_i\alpha,\beta,\gamma$, G_i protein with subunits; GMP, guanine monophosphate $G_q\alpha,\beta,\gamma$, G_q protein with subunits; GTP, guanine triphosphate; IP3, inositol-1,4,5-trisphosphate; K^+_{ATP}-channel, APT-dependent K^+ channel; MT, melatonin; MT_1-R and MT_2-R, melatonin MT_1- and MT_2-receptor; NO, nitrogen monoxide; PDE, phosphodiesterase; PKA, PKC and PKG, protein kinase A, C, and G; PLC, phospholipase C; RORα, RZRβ, and RORγ, nuclear transcription factors RORα, RZRβ, and RORγ.

were suppressible by the control peptide. As originally observed by Balsalobre et al. [138], serum shock leads to induction of clock genes. It was assumed that the roughly 45-kDa-sized major band also represents the nuclear form in INS1 cells, in accordance with the predominant 45 kDa form found in pancreatic tissue. The apparently larger protein in INS1 cells compared to that in pancreas cells might be due to differences in phosphorylation, leading to a greater molecular mass in the β-cell line [139]. The characteristic antiphase expression profiles of *Per1* and *Bmal1*, which were described for the SCN [140], but also for peripheral tissues [141, 142] were also characteristic of the pancreatic oscillator in this study. The *Per1* transcript maximum at zeitgeber time 11 (CT 11) largely overlaps with trough values for *Bmal1* expression at CT 11 and CT 14. This observation is in accordance with the proposed functions of the PERIOD, ARNT, SIM (PAS) transcription factors PER1 and BMAL1 within their respective feedback loops [136, 140]. *Clock* and *Tim* did not show a clear circadian rhythm in the pancreas, which is in accordance with the results of others [143], although some authors [136, 142] reported a low-amplitude circadian rhythm for *Clock* in heart and liver. *Cry1*-transcripts, the translation products of which are known to be the dimerization partner of PER, were reported to be rhythmic in the SCN [144] and heart, although with low amplitude in the heart [145], and they seem to be rhythmic in the pancreas as well according to our results. The *Cry1* and *Per2* expression maxima at night were also observed in the mouse liver [136] - a very similar pattern to that detected in the pancreas. One of the clock-controlled output genes, namely the transcription factor *Dbp* [135], was found to oscillate in rat pancreas with a large amplitude, in accordance with previous results from others in liver, kidney, muscle, lung [141, 146] and heart [142]. Confirming described results, a study on mice SCN from Yamaguchi et al. [147] described *Dbp* oscillation to be in-phase with *Per1* expression. These authors also proposed that *Dpb* plays a role within the circadian core clock mechanism besides its function as an output recipient. Damiola et al. [146] determined circadian *Dbp* expression in whole pancreas extracts and assumed food to be the dominant

zeitgeber for this organ, as well as for the liver. Data, which indicate the expression of *Dbp* within the islet and INS1 cells, also indicate a functional output from an endogenous islet-based oscillator and substantiate the observed cyclic pattern of insulin release from isolated islets [103]. The orphan receptor *Rev-erbα*, which generally acts as a transcriptional repressor, is known to be rhythmically expressed in the SCN and liver as a target of BMAL1/Clock-mediated activation [136]. *Rev-erbα* transcript quantity also varies with a high amplitude in the rat pancreas according to the described data. REV-ERBα, which is supposedly a major circadian regulator of *Bmal1* expression, is concomitantly the recipient of PER-mediated negative output [136]. In accordance with this assumption, *Bmal1* transcripts are at a minimum in the pancreas when the *Rev-erbα* transcription level is at its maximum. In contrast to the central circadian oscillator (CCO) in the SCN, the amplitude of the peripheral oscillators is dampened after a few cycles [148] once removed from the influence of the SCN. Furthermore, the expression pattern of the peripheral oscillators is delayed by several hours compared to the CCO [149]. For the pancreatic islet, the self-sustained rhythm seems to last for several days in an *ex vivo* situation without dampening of the amplitude [103]. Future work will be aimed at elucidating islet-specific entraining cues and their role in regulating islet-linked functions such as insulin and glucagon release [104].

Influence of melatonin on free-radical-induced changes in the rat pancreatic β-cell

For some decades alloxan (ALX) and streptozotocin (STZ) have been used widely to induce diabetes mellitus in animals. Within the last few years, the hypothesis has been established that both compounds lead to selective destruction of pancreatic β-cells through two characteristics: (i) they rapidly accumulate in β-cells where they (ii) induce radical-generating reactions. Pancreatic β-cells are very susceptible to oxidative changes because they possess only low-antioxidative capacity [150]. The redox couple driven by ALX and its reduced derivative dialuric acid produces superoxide anions and hydrogen peroxide, thereby consuming reduced glutathione (GSH) and further weakening the cellular antioxidative defense system [151]. In recent years, evidence has accumulated that reactive oxygen species (ROS) and nitric oxide (NO) contribute to the destruction of pancreatic islets in the pathogenesis of insulin-dependent diabetes mellitus [152]. Comparing data from the literature, it appears that ALX, STZ, ROS generated by xanthine oxidase/hypoxanthine (XO/HX), and NO liberated by different chemicals or cells seem to attack β-cells in a very similar way. All these diabetogens can lead to DNA-strandbreaking, inhibition of various enzymes and membrane leakage [152]. There is no question that melatonin, due to its well-established (and recently re-confirmed) importance as a radical scavenger [153, 154], protects against ALX- as well as STZ-induced diabetes. The literature concerning this topic is extensive. The potential role of melatonin as an antioxidant by scavenging and detoxifying ROS was highlighted by the fact that compounds that are analogues to melatonin can also be used as scavengers, due to their antioxidant properties [155]. Nevertheless, pharmacological doses of melatonin are able to scavenge the high levels of ROS generated in these cases. Also, localized cell-internal melatonin concentrations might be higher. Certainly, melatonin is capable of protecting cells from free-radical damage via its free-radical-scavenging and antioxidant properties [156].

Alloxan diabetes and melatonin

Since generation of ROS is thought to mediate the cytotoxic and diabetogenic action of ALX and since melatonin is an effective scavenger of hydroxyl radicals ($OH^{\bullet}$), it was postulated that melatonin may protect against ALX-induced diabetes in mice [157] and attenuated diabetes-induced alterations in the GSH redox state and in the $OH^{\bullet}$ levels in rabbit [158]. This was also demonstrated at the level of perifused pancreatic islets [159–161]. Furthermore, it was demonstrated by electron spin resonance spectroscopy combined with spin-trapping techniques that $OH^{\bullet}$, generated in the presence of ferrous ions from ALX and GSH, can be effectively scavenged by melatonin. The IC_{50}-value for the scavenging of $OH^{\bullet}$ by melatonin was 23 μmol/L. This value corresponds well with already published results [162]. On the other hand, clearly higher concentrations of melatonin were necessary for half-maximal inhibition of lipid peroxidation (IC_{50} amounted to 0.75 mmol/L) as indicated [163]. Comparable results were published recently [164].

Because of the chain-reaction character of lipid peroxidation, triggered by an attack of $OH^{\bullet}$, it was assumed that the inhibitory concentration of melatonin should be higher than those in spin-trap experiments. This interpretation is supported by a recently published paper in which the authors showed that melatonin is not able to trap peroxyl radicals [165]. The formation of an additional initiator of lipid peroxidation, not identical with $OH^{\bullet}$, and therefore less inhibited or completely uninhibited by melatonin, however, cannot be excluded. On the other hand, it seems fairly certain that neither $O_2^{\bullet-}$ nor H_2O_2 alone are able to initiate lipid peroxidation [166]. These results underline that ALX is able to produce $O_2^{\bullet-}$ as well as H_2O_2 and indicate that the toxicity of ALX appears to be mediated by $OH^{\bullet}$. Thus, melatonin possesses a protective potential against cell injuries induced by $OH^{\bullet}$. This scavenging effect was dependent on the concentration of melatonin in a range of 0.1–1.0 mmol/L. Furthermore, it was shown that the presence of melatonin counteracts the ALX-induced leakage of insulin from pancreatic β-cells and inhibits $OH^{\bullet}$-mediated lipid peroxidation in liposomes. Melatonin also reduces morphological damage of the β-cells after application of ALX [159–161, 163, 167]. Furthermore, in ALX-diabetes, hyperglycemia drives non-enzymatic glycation and oxidation of proteins and lipids, which enhances the formation of advanced glycation end products. Melatonin restored these biochemical abnormalities to normalcy independent of hyperglycemia [168]. On the other hand, ALX-induced diabetes may decrease pineal melatonin synthesis in rats by reducing the activity of hydroxyindole-O-methyltransferase, resulting in a decrease in pineal melatonin secretion [169]. Finally, several scavengers $OH^{\bullet}$

were tested with regard to their efficiency in preventing the transformation of barbituric acid into ALX. Of all the scavengers analyzed, melatonin was shown to be one of the most potent compounds [170].

Streptozotocin diabetes and melatonin

During metabolism of STZ, a variety of toxic intermediates are produced. Beside alkylating agents like methyl cations and methyl radicals [171], it has been shown that ROS are produced by STZ as well [172]. Additionally, STZ liberates NO which has been proposed to be one of the key intermediates of its toxicity [173]. Taken together, STZ-diabetes increases oxidative stress via generation of free radicals [174], lipid peroxidation, levels of malondialdehyde, superoxide dismutase and protein glycosylation [175], decreased levels of catalase and GSH peroxidase [176], as well as DNA single-strand breaks [177]. In the serum of animals with STZ-induced diabetes, melatonin reduces remarkably the degree of both lipid peroxidation [176] and protein glycosylation [175]. Melatonin decreases as well the levels of cholesterol, triglyceride, low-density lipoprotein [178], sialic acid [179], glucose [173, 180], GSH [181] and might regulate the activities of antioxidant enzymes of STZ-diabetic rats [176, 181]. However, the most pronounced effect of the melatonin administration was the prevention of an increase in NO levels in blood plasma during STZ-induced diabetes [182], which implies that melatonin might operate as an NO scavenger and carrier. These findings suggest that melatonin is a strong antioxidant [183], which underlies the beneficial free-radical-scavenging and antioxidant properties [158] and the preservation of β-cell integrity without affecting hyperglycemia [184]. The amazing notion that melatonin was not able to normalize hyperglycemia and/or body weight in STZ-induced diabetes, has been mentioned in many papers [176, 182, 185]. Furthermore, melatonin treatment in STZ-induced type 1 diabetic rats caused a slight increase in the lowered serum insulin concentrations and small partial regeneration/proliferation of β-cells [186]. In contrast, melatonin reduced the hyperinsulinemia associated with type 2 diabetes [187], and may effectively normalize the impaired antioxidant status in STZ-induced diabetes [175, 185]. Interestingly, the recently synthesized derivatives of the melatonin analogue benzimidazole have an inhibitory effect on hydrogen peroxide-induced lipid peroxidation, superoxide dismutase, catalase and glucose-phosphatase dehydrogenase [155] in a comparable manner to melatonin. Melatonin partially or totally restores the STZ-induced consequences of diabetes. STZ-induced diabetes reduced the nocturnal pineal melatonin content in Syrian hamsters [56], but not in rats [58], and the plasma and saliva melatonin levels in type 1 and type 2 diabetic patients [188]. The melatonin rhythm, reflecting the phase of the master clock (nucleus suprachiasmaticus, SCN), however, was not affected by STZ application [189]. This was recently confirmed [190]. The authors found a significant daily rhythm of melatonin concentration changes, not only in the pineal gland and plasma, but also in the pancreas, kidney, spleen and duodenum [190]. However, STZ-induced diabetes resulted in lower melatonin levels in the pancreas, kidney and duodenum compared to the control. The authors speculate that the lower amplitude of melatonin in target organs induced by STZ diabetes might contribute to the desynchronization of daily rhythms and might also lower the antioxidative capacity of tissues [190]. Considering the much lower molar concentration of melatonin compared with vitamin E, it was speculated that melatonin seems to be a more potent antioxidant [178] than vitamin E [191]. Another investigation concluded that the protective effects of melatonin against the β-cell damage caused by STZ may be related to interference with DNA damage and poly(ADP-ribose) polymerase (PARP) activation rather than through effects on NO pathways [192].

What is relevant for humans – clinical implications?

In humans, circulating melatonin shows a circadian rhythm, peaking at night. The rhythm-adjusted mean (mesor) is higher in women than in men, which has been observed in elderly women [193]. The circadian amplitude decreases with age [194] and may be regarded as a marker of the aging process itself [195]. The reduction in melatonin with age may be a factor of increased oxidative damage in the elderly [196], including age-associated neurodegenerative diseases [197]. The mesor and the amplitude of melatonin are modulated by annual variation, the mesor peaking in winter and the amplitude in summer [198]. In this context, the observation that under simulated night-shift conditions, the plasma triacylglycerol response to a standard meal was higher after a nighttime meal than during a daytime meal in the case of a non-adapted nightshift worker, seems to be of interest. In nightshift workers, insulin, glucose and triglycerol were higher after a nighttime meal than after a daytime meal. These disorders are an expression of the desynchronization of bodily functions, combined with a higher incidence of heart diseases [199] and metabolic disturbances like diabetes. To underline this argument, the additional observation that a nocturnal lifestyle is likely one of the health risks to modern man, including night-eating syndrome, obesity and diabetes [200] should be mentioned here. However, there are results which indicate that no alterations in the phase shift [201] are found in the endogenous melatonin rhythm of patients with a metabolic syndrome [202]. Melatonin has been identified not only in the pineal gland, but also in a large number of extrapineal tissues, for example in the pancreas, which is particularly interesting within the scope of the present review. Melatonin has a unique position among the secretory products of the diffuse neuroendocrine system [203]. The wide spectrum of biological activities of melatonin, e.g., the circadian organization of physiological functions, the evidence that melatonin stabilizes and strengthens the coupling of circadian rhythms [204] and, particularly, its main characteristic as a regulator of biological rhythms points to melatonin as an important regulator for the coordination of intercellular interactions [205]. Like melatonin, insulin levels show a circadian rhythm in humans that changes in a opposite manner to melatonin, i.e., insulin showed a maximum when melatonin was at trough level and vice versa [57]. Reductions in

melatonin secretion have been associated with many disorders, for example, with diabetes [94, 106], which implies that the melatonin signal is essential for glucose homeostasis and regulation [204]. In type 2 diabetes, endogenous glucose production and gluconeogenesis display diurnal rhythms that drive the fasting hyperglycemia and are absent in healthy humans. The rise in endogenous glucose production may be related to a deficit in the activity of the SCN in a diabetic situation [206]. In this context, melatonin is likely to play an indirect role in the mechanisms underlying glucose regulation via its actions on the SCN and on sleep regulation [207]. Others voice the opinion that the circadian rhythm of melatonin secretion is blunted in type 2 diabetic patients and there is a complex relationship between various components of the autonomic nervous system and melatonin secretion at night [208]. In addition, it was found that the physiological increase in nocturnal plasma melatonin concentration was not observed in diabetic neuropathies. The impaired melatonin profiles observed in diabetic patients without apparent autonomic neuropathy suggest that a subclinical state of sympathetic denervation may exist in diabetic patients [59]. Furthermore, results of numerous publications support the opinion that the diabetic state enhances the generation of free-radicals and that both melatonin and insulin treatments reduced this oxidative stress. There are suggestions that giving diabetic patients adjuvant therapy with melatonin may have some benefit in controlling diabetic complications [209]. The combined application of melatonin and zinc acetate, when used alone or in combination with the oral hypoglycemic agent metformin, improves impaired fasting and post-prandial glycemic control. Moreover, this treatment decreases the level of glycated hemoglobin [210] and improves diabetes-related complications such as impaired lipid profiles and microalbuminuria in type 2 diabetes [211]. Comparable results were allegedly obtained with the pineal extract epithalamine. Adjuvant use of epithalamine in the treatment of type 2 diabetic patients reportedly promoted a stable compensation of carbohydrate metabolism, by lowering glycosylated hemoglobin and immunoreactive insulin [212]. In conclusion, the reports about interactions between melatonin, the glucose metabolism and diabetes in humans have shown phenomenological and functional causal-analytic results. Molecular and immunocytochemical investigations established the presence of the melatonin receptors MT_1 and MT_2 in human pancreatic tissue [106] and, notably, also in rat islets [105]. Results of a calculation model to determine mRNA expression ratios showed elevated MT_1 receptor expression in comparison to MT_2 expression. The mRNA transcript levels of melatonin receptors appeared to be significantly higher in type 2 diabetic patients than in a control group. An upregulation of receptor expression in type 2 diabetic patients was also observed in immunocytochemical investigations. In addition, the transcription factors RORα, RZRβ, and RORγ indicate increased mRNA expression levels in type 2 diabetic patients. These data demonstrate the existence of the melatonin membrane receptors MT_1 and MT_2 as well as the mRNA expression of nuclear orphan receptors in human pancreatic tissue, with upregulated expression levels in type 2 diabetic patients [106]. This upregulation is combined with both lower plasma melatonin levels and reduced AANAT enzyme activity of pineals, whereas the AANAT mRNA from rat pineals was increased [94].

The insulin receptor mRNA of the pineal gland was found to be reduced in type 2 diabetic rats. These results additionally suggest a functional interrelationship between melatonin and insulin [94]. In this context, recent results are important which reported that melatonin-enhanced insulin-receptor kinase activity increased insulin-receptor substrate-1(IRS-1) phosphorylation, suggesting the potential existence of signaling pathway cross-talk between melatonin and insulin [213]. Furthermore, melatonin also increased the activity of phosphoinositide 3-kinase (PI-3-kinase), whereas the 3′,5′-cyclic adenosine monophosphate-activated protein kinase (AMPK), another important glucose transport stimulatory mediator (via an insulin-independent pathway), was not influenced by melatonin application [214]. In summary, melatonin stimulates glucose transport (to skeletal muscle cells) via the IRS-1/PI-3-kinase pathway, which implies, at the molecular level, a putative role in glucose homeostasis and possibly in diabetes [214]. In addition, the authors speculate that the exposure to light at night and aging, both of which lower melatonin levels, may contribute to the incidence and/or development of diabetes.

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