



Association of melatonin & *MTNR1B* variants with type 2 diabetes in Gujarat population



Roma Patel^a, Nirali Rathwa^a, Sayantani Pramanik Palit^a, A.V. Ramachandran^b, Rasheedunnisa Begum^{a,*}

^a Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, 390002, Gujarat, India

^b Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, 390002, Gujarat, India

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ABSTRACT

Aim/hypothesis: Melatonin is a circadian rhythm regulator and any imbalance in its levels can be related to various metabolic disorders. Melatonin and the genetic variants of Melatonin Receptor 1B (*MTNR1B*) are reported to be associated with Type 2 Diabetes (T2D) susceptibility. The aim of the present study was to investigate i) plasma melatonin levels ii) Single Nucleotide Polymorphisms (SNPs) of *MTNR1B* and iii) Genotype-phenotype correlation analysis in T2D patients.

Methods: Plasma and PBMCs were separated from venous blood of 478 diabetes patients and 502 controls. Genomic DNA was isolated from PBMCs. PCR-RFLP was used for genotyping. Melatonin was estimated from plasma samples by ELISA.

Results: Our study suggests: i) decreased plasma melatonin levels in T2D patients and, ii) association of *MTNR1B* rs10830963 GG genotype with increased Fasting Blood Glucose (FBG).

Conclusion: It can be concluded that reduced titer of melatonin along with altered FBG due to *MTNR1B* genetic variant could act as a potent risk factor towards T2D in Gujarat population.

1. Introduction

A disturbed circadian rhythm is strongly related to Type 2 Diabetes (T2D) and insulin resistance in recent years [1]. Melatonin, a pineal hormone, is known to regulate circadian rhythm and sleep [2]. Melatonin mediates its action through two receptors; MT1 (*MTNR1A*) and MT2 (*MTNR1B*) present in various tissues including pancreatic islets [3,4]. The finding that insulin secretion and plasma melatonin levels are inversely correlated suggests a possible association between melatonin and T2D [5].

It is well-known that T2D is a multifactorial and polygenic metabolic disorder [6]. Substantial variation between different ethnic populations has been reported with regard to the genetic architecture underlying T2D [7,8]. *MTNR1B* (13.16 kb) comprises of two exons, one intron, and 5'- and 3'-flanking regions [9]. Recent studies have identified genetic polymorphisms within *MTNR1B* i.e. rs4753426, rs10830962, and rs10830963 (–1193 C/T, 5' UTR G/C and intron C/G respectively) to be associated with higher fasting glucose levels, impaired insulin secretion, increased risk of T2D and gestational diabetes

in different ethnicities [10,11]. Reduced melatonin levels and an increased melatonin signaling are known to be the risk factors for T2D [12,13].

The aim of this study was to examine whether i) plasma melatonin and polymorphisms in *MTNR1B* (rs4753426 C/T, rs10830962 G/C, and rs10830963 C/G) are associated with T2D in Gujarat population and, ii) the genotype-phenotype correlation of the above-mentioned Single Nucleotide Polymorphisms (SNPs) and plasma melatonin with the metabolic profile are associated with T2D. This is the first genetic association study of *MTNR1B* variants with T2D and metabolic profile in Gujarat population.

2. Materials and methods

2.1. Study subjects

This study was conducted according to the declaration of Helsinki and was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao

Abbreviations: MTNR1A, Melatonin Receptor 1A; MTNR1B, Melatonin Receptor 1B; TC, Total Cholesterol; TG, Triglycerides; LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein; BMI, Body Mass Index; PCR-RFLP, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

* Corresponding author.

E-mail addresses: rasheedunnisa@yahoo.co.in, begum.rasheedunnisa-biochem@msubaroda.ac.in (R. Begum).

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University of Baroda, Vadodara, Gujarat, India (FS/IECHR/2013/1). The importance of the study was explained to all the participants and a written consent was obtained from all diabetes patients and control subjects. The study group included 478 T2D patients (213 males and 265 females) and 502 control subjects (251 males and 251 females). Further, the T2D subjects recruited for the study displayed fasting blood glucose (FBG) levels > 125 mg/dL. BMI (weight kg/height m²) was calculated by recording height and weight.

2.2. Blood collection, DNA extraction, and lipid profiling

Three ml venous blood was drawn from diabetes patients and ethnically matched control subjects between 8:00 AM to 10:00 AM and collected in K₃EDTA coated tubes (Greiner Bio-One, North America Inc., North Carolina, USA). Plasma was separated and stored at –20 °C for evaluation of lipid profile and assay of melatonin. FBG, Total Cholesterol (TC), Triglycerides (TG), and High-Density Lipoprotein (HDL) were estimated by using appropriate commercial kits (Reckon Diagnostics P. Ltd, Vadodara, India). Low-Density Lipoprotein (LDL) was calculated by using Friedewald's (1972) formula. DNA was extracted by phenol-chloroform method and the DNA content and purity were determined spectrophotometrically by 260/280 absorbance ratio. The integrity of DNA was checked electrophoretically on 0.8% agarose gel. The DNA was normalized and stored at 4 °C until further analysis.

2.3. Genotyping of MTNR1B SNPs by PCR-RFLP

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was used to genotype the three *MTNR1B* polymorphisms. The primers used for genotyping are mentioned in Table S1. The reaction mixture (20 µL) included 3.0 µL (150 ng) of genomic DNA, 11.0 µL nuclease-free water, 2.0 µL 10X PCR buffer, 2.0 µL 25 mM dNTPs (Puregene, Genetix Biotech), 1.0 µL of 10 mM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.2 µL (5 U/µL) Taq Polymerase (Puregene, Genetix Biotech). DNA amplification was performed using an Eppendorf Mastercycler gradient (USA Scientific, Inc., Florida, USA). The protocol followed was: initial denaturation at 95 °C for 10 min. followed by 39 cycles of 95 °C for 30 s. (denaturation), primer-dependent annealing (Table S1) for 30 s., extension at 72 °C for 30 s and the final extension at 72 °C for 10 min. 5 µL of the amplified product was checked by electrophoresis on a 2% agarose gel stained with ethidium bromide. Details of the restriction enzymes (Fermentas, Thermo Fisher Scientific Inc., USA) and digested products are mentioned in Table S1. 15 µL of the amplified product was digested with 1U of the corresponding restriction enzyme in a total reaction volume of 20 µL as per the manufacturer's instruction. The digestion products with 50 base pair DNA ladder (Genei Bangalore, India) were resolved on 3.5% agarose gels or 15% polyacrylamide gels stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently) and, further confirmed by sequencing.

2.4. Estimation of plasma melatonin

Plasma levels of melatonin in age and sex-matched patients and controls were measured using human melatonin ELISA Kit (Glory Science Co., Ltd, TX, USA) as per the manufacturer's protocol. The melatonin levels were estimated in the study subjects between the ages of 35–50 years only as the melatonin levels are known to decrease significantly after the age of 50 years.

2.5. Statistical analyses

Evaluation of the Hardy-Weinberg equilibrium (HWE) was

performed for all the polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-squared analysis. The distribution of the genotypes and allele frequencies of *MTNR1B* polymorphisms for patients and control subjects were compared using the chi-squared test with 2 × 2 contingency tables respectively using Prism 5 software (GraphPad software Inc; San Diego CA, USA). P values less than 0.017 for genotype and allele distribution were considered as statistically significant due to Bonferroni's correction for multiple testing. Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. Haplotypes and linkage disequilibrium (LD) coefficients D' = D/Dmax and r² values for the pair of the most common alleles at each site were obtained using <http://analysis.bio-x.cn/myAnalysis.php> [14]. Plasma melatonin levels in patient and control groups were plotted and analyzed by unpaired t-test using Prism 5 software. Association studies of polymorphisms with other parameters were performed using analysis of variance (ANOVA) and Kruskal-Wallis test while correlation analysis was performed using multiple linear regression and spearman's correlation analysis in Prism 5 software after adjusting confounding variables like age and sex. P values less than 0.05 were considered significant for all the association analysis. The statistical power of detection of the association with the disease at the 0.017 level of significance was determined by using the G* Power software.

3. Results

Clinical parameters differed significantly between controls and patients (Table 1). Patients had a significantly higher FBG ($p < 0.0001$). Moreover, obesity factors like BMI, TC, and TG were significantly elevated ($p < 0.0001$, $p = 0.0420$, $p = 0.001$ respectively) while HDL was significantly decreased ($p < 0.0001$) in patients as compared to controls. However, LDL did not differ in the study groups ($p = 0.9322$).

3.1. Association of MTNR1B polymorphisms with T2D

The genotype and allele frequencies of the investigated *MTNR1B* polymorphisms (rs4753426 C/T, rs10830962 G/C, and rs10830963 C/G) are summarized in Table 2 while the representative gel images for PCR-RFLP analysis of *MTNR1B* polymorphisms are shown in Fig. S1. The distribution of genotype frequencies for all the polymorphisms investigated was consistent with Hardy-Weinberg expectations in both patient and control groups ($p > 0.05$).

Furthermore, genotype and allelic frequencies of *MTNR1B* polymorphisms were found to be statistically indifferent ($p > 0.017$) with

Table 1

Baseline characteristics of diabetes patients and controls from Gujarat population.

	Controls (Mean ± SD)	Patients (Mean ± SD)	P value
	(n = 502)	(n = 478)	
Age	39.64 ± 16.35 yr	55.99 ± 10.42 yr	–
Sex: Male		213 (44.5%)	–
	251 (50)%		
Female	251 (50)%	265 (55.5%)	–
Fasting blood glucose (mg/dL)	100.1 ± 7.32	155.3 ± 62.09	< 0.0001
BMI (Kg/m ²)	24.24 ± 5.2	27.04 ± 5.1	< 0.0001
Total Cholesterol (mg/dL)	160.9 ± 42.2	166.2 ± 39.68	0.0420
Triglycerides (mg/dL)	111.7 ± 60.90	164.5 ± 111.1	< 0.001
HDL (mg/dL)	42.79 ± 15.94	38.2 ± 12.6	< 0.0001
LDL (mg/dL)	95.32 ± 41.79	95.10 ± 37.52	0.9322
Onset age (Years)	NA	50.65 ± 10.10	–
Duration of disease (Years)	NA	.06 ± 7.3	–
Family history	NA	64 (14%)	–

Data are presented as Mean ± SD. Statistical significance was considered at $p < 0.05$.

Table 2
Genotype and allele frequency distribution of *MTNR1B* polymorphisms in T2D patients.

Gene/ SNP	Genotype or allele	Controls (Frequency)	Patients (Frequency)	p for Association	Odds ratio	(95% CI)
<i>MTNR1B</i> rs4753426 (–1193 C/T)		(n = 481)	(n = 426)			
	CC	95 (0.20)	102 (0.24)	R	–	–
	CT	252 (0.52)	201 (0.47)	0.0819 ^a	0.7429	0.5312 to 1.039
	TT	134 (0.28)	123 (0.29)	0.4081 ^b	0.8549	0.5896 to 1.240
	C	442 (0.46)	405 (0.48)	0.4983 ^c	0.9381	0.7798 to 1.129
<i>MTNR1B</i> rs10830962 (5' UTR G/C)		(n = 470)	(n = 417)			
	GG	122 (0.26)	114 (0.27)	R	–	–
	GC	226 (0.48)	205 (0.49)	0.8546 ^a	0.9707	0.7065 to 1.334
	CC	122 (0.26)	98 (0.24)	0.4212 ^b	0.8596	0.5946 to 1.243
	G	470 (0.50)	433 (0.52)	0.4198 ^c	0.9261	0.7685 to 1.116
<i>MTNR1B</i> rs10830963 (intron C/G)		(n = 489)	(n = 434)			
	CC	169 (0.35)	133 (0.31)	R	–	–
	CG	259 (0.53)	266 (0.61)	0.0663 ^a	1.305	0.9819 to 1.734
	GG	61 (0.12)	35 (0.08)	0.1902 ^b	0.7291	0.4540 to 1.171
	C	597 (0.61)	532 (0.61)	0.9133 ^c	0.9896	0.8204 to 1.194
	G	381 (0.39)	336 (0.39)			

'n' represents number of samples, 'R' represents reference group, CI refers to confidence interval, ^a, ^b Patients vs controls (genotype) with respect to Reference using chi-square test with 2 × 2 contingency table, ^c Patients vs controls (allele) using chi-square test with 2 × 2 contingency table, Values are significant at p < 0.017 due to Bonferroni's correction.

Bonferroni's correction for multiple testing as shown in Table 2. This study has 95% statistical power for the effect size 0.11, 0.05 and 0.17 to detect association of *MTNR1B* promoter polymorphisms at p < 0.017 in T2D patients and controls.

3.2. Haplotype and linkage disequilibrium analyses of *MTNR1B* polymorphisms

A haplotype evaluation of the three polymorphisms of *MTNR1B* (rs4753426, rs10830962, and rs10830963) was performed and the estimated frequencies of the haplotypes did not differ significantly between patients and controls (global p = 0.681) as shown in Table 3.

The LD analysis revealed that the three polymorphisms investigated in the *MTNR1B* were in low to high LD association (Fig. S2). In particular, rs4753426: rs10830962 (D' = 0.44, r² = 0.18) and - rs4753426: rs10830963 (D' = 0.68, r² = 0.24) showed moderate LD association. rs10830962: rs10830963 showed high LD association (D' = 0.87, r² = 0.47).

3.3. Correlation of *MTNR1B* polymorphisms with FBG, BMI and plasma lipids

Correlation of *MTNR1B* polymorphisms showed that rs10830963 GG genotype was found to be associated with increased FBG (p = 0.02) (Table 4). However, it was not associated (p > 0.05) with BMI and plasma lipids (TC, TG, HDL, LDL). Further, rs4753426 and rs10830962 also did not show any association with FBG, BMI and plasma lipids (p > 0.05).

Table 3
Haplotype frequencies of *MTNR1B* polymorphisms in T2D patients and controls.

Haplotype (<i>MTNR1B</i> rs4753426 C/T, rs10830962 G/C, and rs10830963 C/G)	Patients (Freq. %) (n = 459)	Controls (Freq. %) (n = 501)	p for Association	p (global)	Odd Ratio [95%CI]
CGG	242(0.31)	290(0.325)	0.206	0.681	0.878 [0.719–1.074]
CGC	54(0.069)	47(0.052)	0.242		1.269 [0.849–1.897]
CCC	110(0.141)	130(0.145)	0.511		0.913 [0.696–1.197]
TGC	69(0.088)	71(0.079)	0.717		1.065 [0.755–1.503]
TGG	40(0.051)	39(0.043)	0.608		1.124 [0.716–1.765]
TCC	251(0.321)	291(0.326)	0.408		0.919 [0.753–1.122]

'CI' represents confidence interval (Frequency < 0.03 in both case and control has been dropped and was ignored in the analysis).

3.4. Plasma melatonin levels and their correlation with FBG, BMI and plasma lipids

Plasma melatonin levels monitored in 37 controls and 45 patients showed a significant decrease (p = 0.001) in T2D patients as compared to controls (Fig. 1).

Spearman's Correlation analysis revealed that there is no correlation between melatonin levels and BMI, FBG and plasma lipids (R² = 0, p > 0.05) (Table 5).

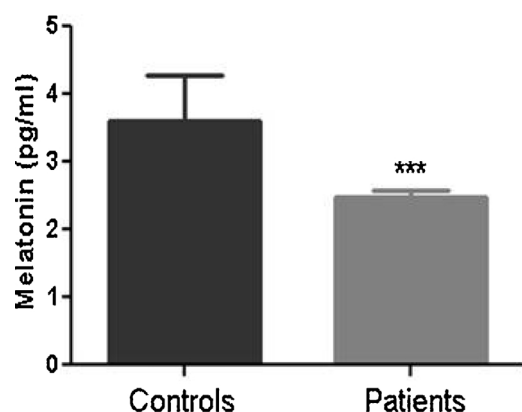
4. Discussion

Disturbances in sleep have been implicated in the dysregulation of blood glucose levels and have also been reported to increase the risk of T2D and diabetes complications. Melatonin, a pineal hormone regulates energy metabolism by maintaining circadian rhythms [15]. Genome-wide association studies have shown a close association between *MTNR1B* polymorphisms and fasting hyperglycemia and T2D [16].

Our study on genetic variants of *MTNR1B* (rs4753426, rs10830962, and rs10830963), melatonin levels and their correlation with BMI, FBG, and plasma lipid profile in T2D patients show that *MTNR1B* polymorphisms are not associated with T2D and obesity-related traits but rs10830963 GG genotype has a significant association with elevated FBG. Similarly, an association of this genotype with FBG has been shown in both South Indian and Han Chinese population [17,18]. However, this SNP does not show any correlation with obesity-linked parameters in T2D [19]. Moreover, several studies in different populations have found no association between *MTNR1B* polymorphisms and T2D [19,20,21]. Interestingly, a large-scale meta-analysis when

Table 4Genotype-phenotype correlation analyses of *MTNR1B* polymorphisms with BMI, FBG and plasma lipid profile.

Genotype/ Allele	FBG (mg/dL)	BMI (Kg/m ²)	Triglycerides (mg/dL)	Total Cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
<i>MTNR1B</i> rs4753426						
CC	134.3(59.98)	25.35(5.46)	131.5(87.05)	164.8(42.63)	40.74(12.77)	97.76(40.26)
CT	129.1(52.01)	25.71(5.51)	138.8(96.98)	162.9(41.07)	41.65(16.73)	93.45(41.17)
TT	133.5(62.24)	25.72(5.04)	142.0(85.58)	161.9(39.62)	38.96(12.10)	94.54(36.65)
P value	0.3272	0.5775	0.1710	0.8221	0.2053	0.5263
<i>MTNR1B</i> rs10830962						
GG	133.9(61.83)	25.24(5.46)	138.6(89.46)	138.6(89.46)	39.75(13.10)	93.32(38.72)
GC	130.9(54.49)	25.79(5.43)	140.7(101.40)	140.7(101.40)	41.48(16.57)	94.37(40.69)
CC	131.4(57.66)	25.41(5.22)	134.9(83.62)	134.9(83.62)	39.91(12.81)	97.38(40.83)
P value	0.5599	0.4869	0.9440	0.9440	0.9170	0.8120
<i>MTNR1B</i> rs10830963						
CC	130.7(61.79)	25.33(5.05)	134.8(91.39)	162.5(39.90)	40.60(13.15)	94.98(37.13)
CG	133.4(52.74)	25.82(5.40)	140.7(95.14)	163.2(41.56)	40.64(16.14)	94.40(41.06)
GG	160.6(65.94)	24.63(5.99)	136.6(90.98)	170.6(43.85)	40.82(11.98)	102.5(42.65)
P value	0.0029	0.0528	0.7865	0.3515	0.4910	0.3895

Data are presented as Mean \pm SD. Statistical significance was considered at $p < 0.05$.**Fig. 1.** Plasma melatonin levels in controls and patients. Comparison of findings showed a significant decrease in the melatonin levels in patients compared to controls ($p < 0.001$).**Table 5**

Correlation analysis of plasma melatonin with BMI, FBG, and plasma lipids.

	Melatonin	
	R ²	p
BMI (Kg/m ²)	0.01	0.44
FBG (mg/dL)	0.11	0.34
Triglycerides (mg/dL)	0.00	0.81
Total Cholesterol (mg/dL)	0.03	0.20
HDL (mg/dL)	0.01	0.45
LDL (mg/dL)	0.06	0.10

R² = Coefficient of correlation, r = Spearman's correlation coefficient [$p < 0.05$, significant; $p > 0.05$, non significant].

stratified according to the ethnicity revealed that *MTNR1B* rs10830963 is associated with T2D in Caucasians but not in Asians [22]. Our observation of association of G allele with FBG stands substantiated by a meta-analysis [23] and a GWAS study [16,24]. However, they also found an association with increased T2D risk which is not seen in our study. Further, Takeuchi et al. have shown rs10830963 polymorphism to be associated with elevated FBG in two Asian populations [25]; South Asian Sri Lankans and East Asian Japanese. The effect of the G allele on FBG was found in children and adolescents indicating an early impact of this SNP during development [26,27]. Our study also shows a clear correlation between lowered melatonin levels and T2D risk, a finding supported by the observations of Peschke et al. [28] and McMullan et al. [29]. Although the location of the rs10830963 variant

is in an intron, an un-conserved genomic region, its association with increased *MTNR1B* expression in islets, FBG and T2D stand well documented.

Haplotype analysis of the three polymorphic sites of *MTNR1B* reveals that none of the *MTNR1B* haplotypes have an association with T2D risk. Further, LD analysis suggests that *MTNR1B* variants rs4753426 and rs10830963 have strong LD association demonstrating a high linkage between these loci. We did not find any correlation between melatonin levels and FBG, BMI or plasma lipids. Yildiz et al. have also reported that melatonin does not show any correlation with any of the anthropometric and biochemical parameters [30].

The *MTNR1B* risk allele genotype (GG) has been associated with insulin resistance [31,13] and an increase in *MTNR1B* expression by 2–4 fold in human pancreatic islets [13]. Gaulton et al. revealed that the G allele of rs10830963 favorably binds to NEUROD1 in islet cells further increasing FOXA2-bound enhancer activity and *MTNR1B* expression leading to increased risk of T2D [32]. Our observation of association of *MTNR1B* risk genotype with higher FBG could be attributed to reduced insulin levels probably through the *MTNR1B* mediated reduced cAMP levels in pancreatic beta cells in keeping with the reported effects of *MTNR1B* induced inhibition of insulin secretion [13,33]. Moreover, our previous study has shown an association between *NPY* polymorphism, and related elevation in *NPY* levels, and T2D [34]. In addition, *NPY* has been reported to mimic the potential of light to suppress melatonin secretion [35]. Apparently, the elevated *NPY* levels in our T2D patients and decreased melatonin levels and *MTNR1B* variant could together account for elevated FBG and T2D risk as shown in Fig. 2.

5. Conclusion

T2D patients showed reduced melatonin levels and association of *MTNR1B* rs10830963 GG genotype with increased FBG levels, suggesting an important link between melatonin, *MTNR1B*, and FBG in T2D. For the first time, we report a possible involvement of melatonin and its receptor gene variant conferring a risk towards T2D in Gujarati population.

Author contributions

RB conceived the idea and designed the experiments. RP performed the experiments. RP, SP, and NR contributed to data acquisition. Data analysis and manuscript writing were performed by RP. RB and AVR did critical revision and approved the manuscript.

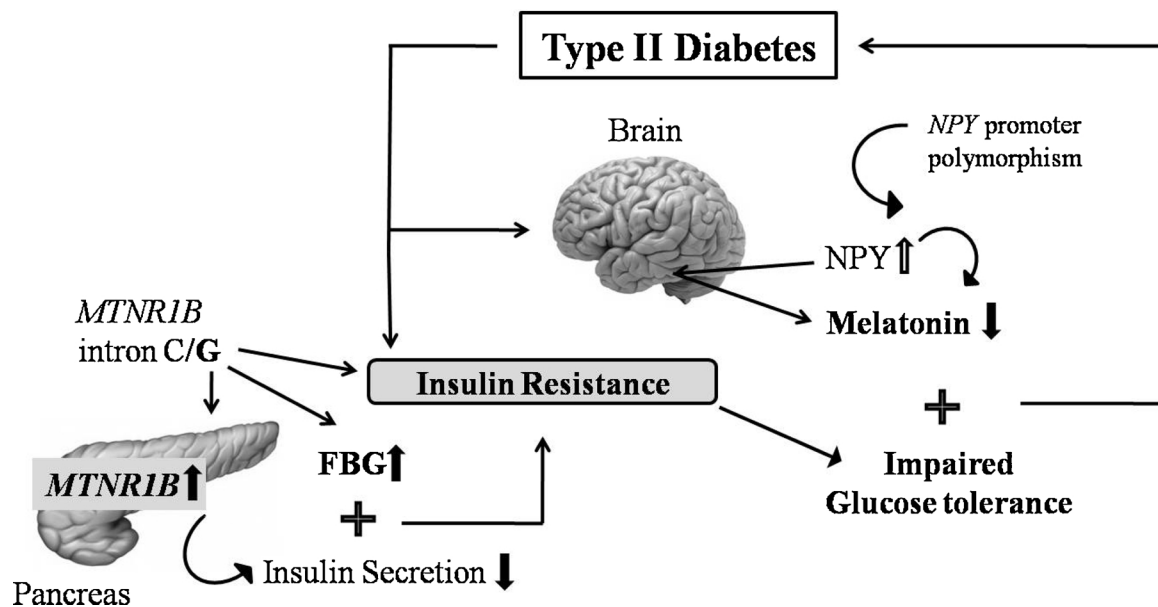


Fig. 2. Role of melatonin and its receptor genetic variant in T2D susceptibility. *NPY* and *MTNR1B* polymorphisms might modulate their respective gene expression. A rise in *NPY* levels might lead to a reduction in melatonin levels and *MTNR1B* rs10830963 GG genotype might increase FBG levels. The increased *MTNR1B* expression could also contribute to impaired glucose tolerance by reducing insulin secretion. Thus, altogether decreased melatonin along with its receptor genetic variant confers risk towards T2D.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2018.04.058>.

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