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Common variant in *MTNR1B* associated with increased risk of type 2 diabetes and impaired early insulin secretion

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Genome-wide association studies have shown that variation in *MTNR1B* (melatonin receptor 1B) is associated with insulin and glucose concentrations. Here we show that the risk genotype of this SNP predicts future type 2 diabetes (T2D) in two large prospective studies. Specifically, the risk genotype was associated with impairment of early insulin response to both oral and intravenous glucose and with faster deterioration of insulin secretion over time. We also show that the *MTNR1B* mRNA is expressed in human islets, and immunocytochemistry confirms that it is primarily localized in β cells in islets. Nondiabetic individuals carrying the risk allele and individuals with T2D showed increased expression of the receptor in islets. Insulin release from clonal β cells in response to glucose was inhibited in the presence of melatonin. These data suggest that the circulating hormone melatonin, which is predominantly released from the pineal gland in the brain, is involved in the pathogenesis of T2D. Given the increased expression of MTNR1B in individuals at risk of T2D, the pathogenic effects are likely exerted via a direct inhibitory effect on β cells. In view of these results, blocking the melatonin ligand-receptor system could be a therapeutic avenue in T2D.

T2D incidence and prevalence are increasing at an alarming rate worldwide. It is well established that T2D is multifactorial and that multiple genes and environmental and behavioral factors combine to cause the disease. Recent genome-wide association studies (GWAS) have provided new insights into the nature of these genetic factors[1–5]. Many of the T2D-associated variants identified in these studies seem to influence the capacity of β cells to cope with increased insulin demands imposed by insulin resistance. One of the GWAS (Diabetes Genetics Inititative; DGI) also provided information on association with 18 quantitative traits[1], including measures of insulin secretion and action. One of the strongest signals for glucose-stimulated insulin secretion in the DGI scan emanated from a SNP (rs10830963) in *MTNR1B* on chromosome 11 ($P = 7 \times 10^{-4}$, rank order 595). Given that the melatonin pathway had previously been suggested to be involved in pathogenesis of T2D, the *MTNR1B* gene was a prime candidate gene for T2D. This SNP was also strongly associated ($P = 3.2 \times 10^{-50}$) with elevated fasting glucose concentrations in a meta-analysis of the recent GWAS of T2D[6].

Melatonin is a circulating hormone predominantly secreted from the pineal gland, although other endocrine cell systems may also synthesize and release this hormone[7], which then could exert hitherto unknown autocrine and paracrine effects[8]. Melatonin is an

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Table 1 Samples used in this study

Study	*N* (with diabetes)	Geographic origin	Age (y)	BMI (kg/m^2)
Malmoe Preventive Project (MPP)	16,061 (2,063)	Sweden	45.5 ± 6.9	24.3 ± 3.3
Botnia PPP	3,300	Finland	48.5 ± 15.9	26.1 ± 4.2
Botnia prospective cohort	2,770 (138)	Finland	44.9 ± 14.2	25.6 ± 4.1
Helsinki Birth Cohort	1,600	Finland	61.6 ± 3.0	27.1 ± 4.3
FUSION	522	Finland	39.1 ± 12.2	26.0 ± 6.4
METSIM	4,369	Finland	59.3 ± 2.8	26.9 ± 3.8

Data are shown as mean ± s.d.

indoleamine formed from tryptophan via acetylation and subsequent methylation of the neurotransmitter serotonin. It has primarily been implicated in the regulation of circadian rhythms, and circulating levels of the hormone are high during night and drop during daylight[7]. In fact, it has been proposed that melatonin could be involved in a circadian lowering of nocturnal insulin levels[9]. Effects of melatonin are mediated by two distinct receptors, MTNR1A and MTNR1B[10], which are members of the G protein–coupled receptor family, specifically inhibitory G proteins (G_i). Both receptors have been found to be expressed in human and rodent islets[11], with MTNR1A predominating, especially in glucagon-producing α cells[12]. There is some evidence that melatonin may exert an effect on insulin secretion, in that acute effects exerted by cAMP-elevating agents are inhibited by melatonin, whereas prolonged effects of the hormone may be stimulatory[7]. Here we provide new evidence that the common variant rs10830963 in the *MTNR1B* gene—or a variant(s) in linkage disequilibrium with it—increases risk of future T2D by causing impaired early insulin secretion. Further, we present functional data that suggest a potential role of the melatonin system, in particular the MTNR1B receptor, in regulation of glucose homeostasis in man.

First, we studied whether the *MTNR1B* rs10830963 SNP predicts future T2D in 16,061 Swedish (from the Malmoe Preventive Project, MPP) and 2,770 Finnish (from the Botnia study) subjects, 2,201 (2,063 + 138) of whom developed diabetes during a median follow-up period of 23.5 years (**Table 1**). The frequency of the risk G allele of SNP rs10830963 was higher in individuals from the MPP study who converted to T2D compared to nonconverters (30.2% versus 28.0%, $P = 0.002$). This yielded a modestly increased risk of 1.12 (95% CI = 1.04–1.20, $P = 0.002$). There was no significant difference between converters and nonconverters in the Botnia study, but here only 138 individuals developed T2D during a 7-year follow-up period (31.0% versus 29.3%; OR = 1.09, 95% CI = 0.82–1.43, $P = 0.56$). In the combined analysis of the two cohorts, the risk allele was associated with a 1.11-fold increased risk of future T2D (95% CI = 1.03–1.18, $P = 0.004$). This relatively modest risk for future T2D probably explains why this SNP was not identified as being associated with T2D in previous GWAS (OR = 1.12 (95% CI = 1.04–1.20), $P = 0.003$ in DIAGRAM). However, the effect on glucose levels seems much stronger; in nondiabetic individuals from the MPP study, rs10830963[G] carriers had a higher fasting plasma glucose concentration at baseline (CC: 5.38 ± 0.54 mmol/l, CG: 5.44 ± 0.55 mmol/l, GG 5.50 ± 0.55 mmol/l, $P = 3 \times 10^{-19}$), which remained elevated throughout the 25-year follow-up period (CC: 5.41 ± 0.54 mmol/l, CG: 5.49 ± 0.54 mmol/l, GG 5.55 ± 0.54 mmol/l, $P = 2 \times 10^{-31}$) (**Fig. 1a**).

Next, we examined insulin secretion in 3,300 nondiabetic participants from the population-based Botnia PPP study. We observed a dose-dependent decrease (corrected early insulin response to glucose (CIR): beta = –0.170 ± 0.021, $P = 5 \times 10^{-16}$; disposition index (DI): beta = –0.241 ± 0.022, $P = 1 \times 10^{-26}$) with increasing number of G alleles of rs10830963 (**Table 2** and **Fig. 1b,c**). These findings were replicated in the METabolic Syndrome In Men (METSIM) study, where both CIR (beta = –0.143 ± 0.022, $P = 1 \times 10^{-10}$) and DI (beta = –0.128 ± 0.022, $P = 9 \times 10^{-9}$) were associated with rs10830963 in 4,257 subjects.

In the Botnia prospective study, 2,328 nondiabetic carriers of rs10830963[G] showed lower insulin secretion at baseline (CIR: beta = –0.160 ± 0.026, $P = 6 \times 10^{-10}$; DI: beta = –0.171 ± 0.026, $P = 9 \times 10^{-11}$), which was maintained lower throughout the 7-year follow-up period (CIR: beta = –0.188 ± 0.026, $P = 1 \times 10^{-12}$; DI: beta = –0.179 ± 0.029, $P = 8 \times 10^{-10}$) (**Fig. 1d**). Further, rs10830963[G] was also associated with impaired insulin secretion during an intravenous glucose tolerance test in 505 nondiabetic individuals from the Botnia study (FPIR: beta = –0.065 ± 0.023, $P = 0.004$; **Fig. 1e**). rs10830963[G] was also associated with reduced acute insulin response to glucose (AIR: $P = 2.2 \times 10^{-6}$; DI: $P = 5.0 \times 10^{-3}$) in 522 nondiabetic individuals from the FUSION study[13] (**Table 2**).

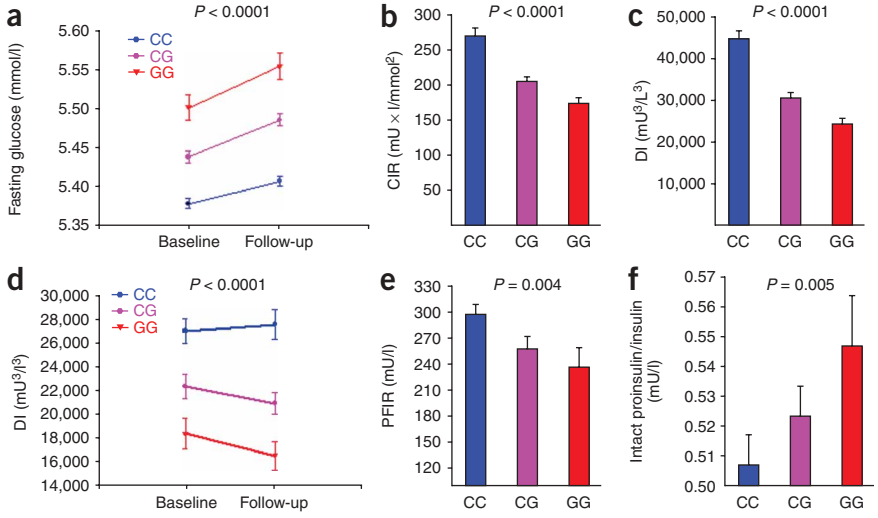

Figure 1 Insulin secretion according to different *MTNR1B* rs10830963 genotypes. (**a**) Change in fasting plasma glucose concentrations during 24-year follow-up in nondiabetic subjects (Malmoe study, *N* = 13,674). (**b**) Corrected early insulin response to glucose (CIR) during OGTT (Botnia PPP cohort; *N* = 3,300). (**c**) Disposition index (DI) represents early insulin response to glucose corrected for insulin sensitivity by the Matsuda index (CIR × ISI, Botnia PPP cohort; *N* = 3,300). (**d**) Change in insulin secretion (disposition index) over time in nondiabetic subjects (Botnia prospective cohort, *N* = 2,328). (**e**) Insulin secretion measured as first-phase insulin response during an IVGTT (Botnia cohort; *N* = 505). (**f**) Intact proinsulin-to-insulin ratio in the fasting state (Helsinki Birth Cohort, *N* = 1,600). Bars represent mean ± s.e.m. Blue lines represent nonrisk and red lines risk genotype carriers of rs10830963 in *MTNR1B*.

Finally, we examined whether the SNP would influence proinsulin processing as reflected in the ratio between proinsulin and insulin in 1,600 nondiabetic participants of the Helsinki Birth Cohort Study[14]. Also here, carriers of the *MTNR1B* risk genotype had impaired early insulin response to oral glucose (CIR: beta = –0.109 ± 0.027, $P = 5 \times 10^{-5}$; DI: beta = –0.122 ± 0.027, $P = 8 \times 10^{-6}$; **Table 2**). In addition, risk allele carriers had an elevated intact proinsulin-to-insulin ratio ($P = 0.005$; **Table 2** and **Fig. 1f**). However, an increased proinsulin-to-insulin ratio does not a priori imply a specific defect in proinsulin processing, as proinsulin concentrations rise under most conditions of stressed β cells.

The melatonin 1 B receptor (MTNR1B) is expressed in human islets and in β cells. Using quantitative RT-PCR (Taqman), we observed that both MTNR1A and MTNR1B were expressed in human islets as well as in clonal β cells. In contrast to previous findings[11,12], both receptors were expressed at near equal level in human islets. Moreover, islet expression of MTNR1B was confirmed by immunocytochemistry (**Fig. 2**). Again, in contrast to a previous report, where single-cell PCR identified *MTNR1A* mRNA primarily in α cells[12], we observed expression of MTNR1B predominantly in β cells in both human and rodent islets (**Fig. 2**). MTNR1A was also observed in islets; its expression was

Table 2 Effect of the *MTNR1B* rs10830963 on insulin secretion in the studied cohorts

		Genotypes				Additive model		
Study	Phenotype	CC	CG	GG	RA	BETA	s.e.m.	*P* value
DGI WGAS (OGTT $n = 1{,}020$)	Age (y)	59 ± 10	59 ± 10	58 ± 10		–		0.74
	BMI (kg/m^2)	26.5 ± 3.6	26.7 ± 4.0	27.3 ± 3.7		–		0.14
	Fasting P-glucose (mmol/l)	5.28 ± 0.53	5.32 ± 0.52	5.38 ± 0.60	0.31	0.045	0.022	0.039
	CIR (mU × l/mmol^2)	180 ± 360	165 ± 1,912	144 ± 163		–0.166	0.048	7×10^{-4}
	DI (mU^3/l^3)	24,036 ± 29,445	20,285 ± 27,763	16,555 ± 22,974		–0.173	0.046	2×10^{-4}
Botnia PPP (OGTT $n = 3{,}300$)		CC	CG	GG				
	Age (y)	48.3 ± 16.0	48.5 ± 15.9	49.6 ± 15.9		–		0.38
	BMI (kg/m^2)	26.12 ± 4.21	26.22 ± 4.22	26.19 ± 3.82		–		0.79
	Fasting P-glucose (mmol/l)	5.06 ± 0.54	5.25 ± 0.55	5.28 ± 0.55	0.30	0.134	0.014	2×10^{-22}
	CIR (mU × l/mmol^2)	271 ± 415	205 ± 245	175 ± 134		–0.170	0.021	5×10^{-16}
	DI (mU^3/l^3)	44,631 ± 87,537	30,499 ± 49,947	24,316 ± 21,582		–0.241	0.022	1×10^{-26}
Botnia prospective (OGTT $n = 2{,}328$)	Baseline	CC	CG	GG				
	Age (y)	45.8 ± 13.2	45.1 ± 13.8	45.6 ± 14.2		–		0.52
	BMI (kg/m^2)	25.5 ± 4.1	25.7 ± 3.7	25.7 ± 3.8		–		0.48
	Fasting P-glucose (mmol/l)	5.47 ± 0.57	5.55 ± 0.57	5.64 ± 0.54	0.29	0.081	0.019	1×10^{-5}
	CIR (mU × l/mmol^2)	176 ± 183	150 ± 164	129 ± 137		–0.160	0.026	6×10^{-10}
	DI (mU^3/l^3)	26,958 ± 34,304	22,340 ± 31,320	18,375 ± 17,416		–0.171	0.026	9×10^{-11}
	Follow-up	CC	CG	GG				
	Age (y)	53.8 ± 13.8	52.7 ± 14.3	53.3 ± 14.9		–		0.25
	BMI (kg/m^2)	26.5 ± 4.1	26.7 ± 4.2	26.7 ± 4.2		–		0.41
	Fasting P-glucose (mmol/l)	5.25 ± 0.56	5.34 ± 0.56	5.41 ± 0.61		0.086	0.019	5×10^{-6}
	CIR (mU × l/mmol^2)	234 ± 238	188 ± 192	145 ± 125		–0.188	0.026	1×10^{-12}
	DI (mU^3/l^3)	27,508 ± 40,934	20,888 ± 27,012	16,502 ± 16,261		–0.179	0.029	8×10^{-10}
		CC	CG	GG				
Helsinki Birth Cohort (OGTT $n = 1{,}600$)	Age (y)	61.6 ± 3.0	61.5 ± 3.0	61.6 ± 3.1		–	–	0.96
	BMI (kg/m^2)	27.0 ± 4.2	27.2 ± 4.4	27.1 ± 4.2		–	–	0.53
	Fasting P-glucose (mmol/l)	5.41 ± 0.55	5.55 ± 0.56	5.59 ± 0.53	0.34	0.096	0.019	3×10^{-7}
	CIR (mU × l/mmol^2)	209 ± 196	175 ± 150	177 ± 188		–0.109	0.027	5×10^{-5}
	DI (mU^3/l^3)	19,646 ± 21,504	15,552 ± 15,063	15,699 ± 17,881		–0.122	0.027	8×10^{-6}
	Intact proinsulin/insulin	0.51 ± 0.26	0.52 ± 0.26	0.55 ± 0.24		0.024	0.009	0.005
METSIM ($n = 4{,}257$)	Age (y)	59.3 ± 5.8	59.4 ± 5.8	59.1 ± 5.7	0.36	–	–	–
	BMI (kg/m^2)	26.9 ± 3.9	26.9 ± 3.7	26.5 ± 3.7		–0.058	0.020	4.3×10^{-3}
	Fasting P-glucose (mmol/l)	5.6 ± 0.5	5.7 ± 0.5	5.8 ± 0.5		0.165	0.022	9.4×10^{-14}
	CIR (mU × l/mmol^2)	196 ± 212	168 ± 165	152 ± 143		–0.143	0.022	1.3×10^{-10}
	DI (mU^3/l^3)	21,554 ± 28,426	17,878 ± 18,235	16,798 ± 16,461		–0.128	0.022	9.8×10^{-9}
Botnia (IVGTT $n = 505$)		CC	CG	GG				
	FPIR	297 ± 195	259 ± 194	237 ± 139	0.27	–0.065	0.023	0.004
FUSION (FSIGT $n = 522$)	AIR (pM × 8 min)	2,632 ± 1,731	2,064 ± 1,468	1,554 ± 1,092	0.35	–0.316	0.067	2×10^{-6}

Data are shown as means ± s.d. CIR, corrected early insulin response to glucose during OGTT; DI, disposition index; FPIR, first-phase insulin response during IVGTT; AIR, acute insulin response during frequently sampled intravenous glucose tolerance test (FSIGT); RA, risk allele.

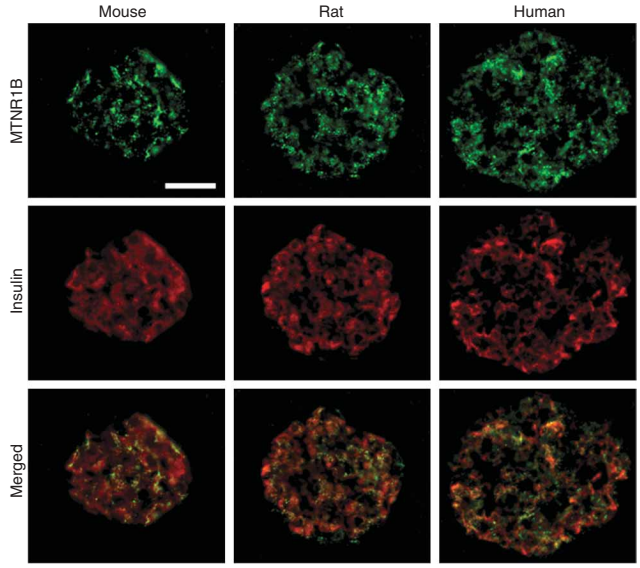

Figure 2 Colocalization of MTNR1B and insulin protein in mouse, rat and human pancreatic islets. Scale bar, 50 μm.

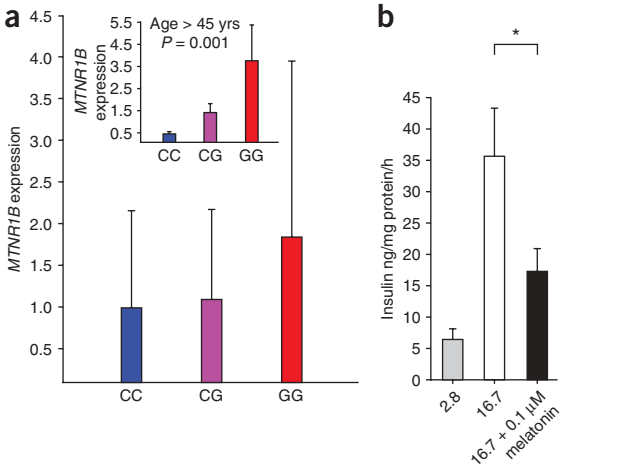

Figure 3 Expression of *MTNR1B* in human pancreatic islets. (**a**) The *MTNR1B* mRNA levels were higher in risk GG genotype carriers (total $n = 51$, CC = 21, CG = 25, GG = 5; nonadjusted $P = 0.25$, age-adjusted $P = 0.01$). The insert graph shows expression of the *MTNR1B* mRNA levels in the individuals above mean age of 45 years (total $n = 25$, CC = 10, CG = 13, GG = 2; $P = 0.001$): the *MTNR1B* mRNA levels were higher in risk GG genotype carriers. (**b**) Insulin secretion in INS-1 832/13 clonal β-cells in response to stimulation with 2.8 mM (gray bar) and 16.7 mM glucose (white bar) in with the presence and absence of 0.1 μM melatonin (black bar). Individual experiments were done in triplicate ($n = 7$, $^*P < 0.037$). Bars represent mean ± s.e.m.

less abundant and seemed to be restricted to a population of peripherally located β cells in human, mouse and rat islets.

Next, we analyzed whether islet expression of MTNR1B, which we now had established in β-cells, correlated with presence of rs10830963[G] in the *MTNR1B* gene as well as with T2D. To this end, we used both quantitative RT-PCR and microarray. Using RT-PCR, we found that individuals carrying the G allele showed higher expression of MTNR1B as compared with carriers of the C allele (age-adjusted $P = 0.01$, **Fig. 3a**). Notably, this effect was almost exclusively seen in individuals older than 45 years ($P = 0.001$, **Fig. 3a** insert). The microarray experiments (Affymetrix HU 133) were done on islets isolated from four nondiabetic and four T2D islet donors[15]. There was a trend toward higher expression of MTNR1B in T2D than in nondiabetic islets ($P = 0.20$, **Supplementary Fig. 1a** online), and expression correlated inversely with glucose-stimulated insulin secretion (**Supplementary Fig. 1b**).

To determine the effects of melatonin on insulin secretion, we acutely incubated clonal β cells (832/13) at low and high glucose concentrations in the presence of 0.1 μM melatonin. Addition of melatonin exerted a clear inhibitory effect on insulin secretion provoked by glucose (**Fig. 3b**).

The present findings provide strong support for a role of melatonin and its receptor MTNR1B in the pathogenesis of T2D. A common variant in the MTNR1B receptor was associated with an increase in fasting glucose over time and predicted future T2D, most likely through impairment of insulin secretion from the pancreatic β-cell function[7]. Notably, this effect became more pronounced with increasing age, most likely as a consequence of the increased demands imposed by increased age-related insulin resistance. This effect can be understood in light of what is known about the function of melatonin in islets based on previous studies as well as our present results. The MTNR1B is coupled to an inhibitory G protein[10]. Activation of MTNR1B by melatonin would therefore block activation of adenylate cyclase, which is the predominant mode of action for incretin hormones, such as GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), both of which raise intracellular cAMP. There is also evidence supporting that glucose stimulation of the β cell by itself leads to a rise in intracellular cAMP. Indeed, it has previously been observed that addition of melatonin blocks cAMP formation in β cells[16]. Here, we confirmed previous observations, although discrepant results have been reported[12], that melatonin acutely blocks glucose-induced insulin secretion[7]. Thus, in a situation where expression of MTNR1B is increased, it could be anticipated that cellular cAMP levels will be lower. Hence, the potentiating effect that this nucleotide exerts on insulin secretion, via mechanisms both dependent on and independent of protein kinase A, would be diminished, leading to impaired insulin secretion. This potential pathogenic situation would be further aggravated if melatonin levels are elevated. In fact, this seems to be the case: studies have reported that the circadian rhythm in melatonin secretion is perturbed in T2D[17]. It has been suggested that secretion of the hormone is elevated during the day, when it normally should be low, which could lead to reduced insulin secretion.

There are therapeutic implications of our findings. First, if melatonin has a negative role in the development of T2D, antagonists of the receptors targeted to β cells could be of utility. Second, individuals with the risk profile conferred by the *MTNR1B* rs10830963 SNP may be less responsive to treatment with GLP-1 analogs as well as inhibitors of GLP-1 degradation (DPP-IV inhibitors). Identifying these individuals may allow tailoring of a more precise therapy in T2D.

Our findings lend support to earlier reports of a role of the melatonin system for islet function and also provide new insights into the mechanisms by which the system may play a role in the pathogenesis of T2D. Interfering with its action may be a new therapeutic avenue in T2D.

METHODS

Study populations. In the Malmoe Preventive Project (MPP), 33,346 Swedish subjects (22,444 men and 10,902 women; mean age 49 years, 24.5% with

impaired fasting (IFG) and/or impaired glucose tolerance (IGT)) from the city of Malmoe in southern Sweden participated in a health screening during 1974–1992 (ref. 18). All individuals underwent a physical examination and blood was drawn for measurements of fasting blood glucose and lipid concentrations. In addition, 18,900 consecutively enrolled persons also had an oral glucose tolerance test (OGTT). Information on lifestyle factors and medical history was obtained by questionnaire. Of individuals participating in the initial screening 4,931 are deceased and 551 are lost from follow-up. Of the eligible individuals, 25,000 were invited to a rescreening visit during 2002–2006, which included a physical examination and fasting blood samples for measurements of plasma glucose and lipids. Of the invited subjects, 17,284 persons participated in the rescreening. Of them 1,223 were excluded because of lacking information or DNA (or T2D at baseline)[19]. Thereby, 16,061 nondiabetic subjects, 2,063 of whom developed T2D, were included in the current analyses. Diagnosis of diabetes was confirmed from subject records or on the basis of a fasting plasma glucose concentration greater than 7.0 mmol/l.

The Botnia study started in 1990 at the west coast of Finland aiming at identification of genes' increasing susceptibility to T2D in members from families with T2D. The prospective part included 2,770 nondiabetic family members and/or their spouses (1,263 men and 1,507 women, mean age 45 years), 138 of whom developed T2D during a 7.7 year (median) follow-up period[19–21]. All subjects were given information about exercise and healthy diet and exposed at 2- to 3-year intervals to a new OGTT.

Prevalence, Prediction and Prevention of T2D (PPP Botnia) study is a population-based study in the Botnia region which included approximately 10% of the population aged 18–74 years (mean age 51 ± 17 years.) Diagnosis of diabetes was confirmed from subject records or on the basis of a fasting plasma glucose concentration greater than 7.0 mmol/l and/or 2 h glucose greater than 11.1 mmol/l. Of the nondiabetic individuals, 2,328 also had serum insulin concentrations measured at baseline and during follow-up.

The Finland–United States Investigation of Non-insulin-dependent Diabetes Mellitus Genetics (FUSION) study has been described in detail[2,13]. For this study 578 nondiabetic spouses or offspring were included in the study of insulin response to intravenous glucose using tolbutamide-modified frequently sampled intravenous glucose tolerance tests (FSIGTs)[22,23] and analyzed by the Minimal Model method[24] to derive quantitative measures of insulin sensitivity (S_I) and glucose effectiveness (S_G). Insulin secretion was assessed as the acute insulin response to glucose (AIR) as described by Ward *et al.*, and beta-cell function was assessed using the disposition index ($DI = S_I \times AIR$)[25].

The Helsinki Birth Cohort Study (HBCS) has been previously described. In the present study, 1,600 nondiabetic subjects (698 men and 902 women, mean age 62 ± 3 years) were included[14]. In 2001–2004 all subjects participated in a clinical examination, including a standard 75 g OGTT. Intact proinsulin concentration was measured at 0 min and the fasting proinsulin/insulin ratio (PI/I) was calculated.

The METabolic Syndrome In Men (METSIM) study includes men aged 45–70 years, randomly selected from the population of the town of Kuopio, Eastern Finland, Finland (population 95,000). The present analysis is based on the first 4,386 nondiabetic subjects examined for METSIM with available OGTT data. Samples for the OGTT were obtained at fasting and at 30 and 120 min postload. The CIR and ISI were calculated from OGTT glucose and insulin data as described below.

All participants from the different studies gave informed consent and the local ethics committees approved the protocols.

Measurements. Weight, height and waist and hip circumferences were measured as previously reported[18,19]. In the MPP cohort at baseline, blood samples were drawn at 0, 40 and 120 min of the 75 g OGTT for measurements of blood glucose and serum insulin concentrations, and fasting samples were drawn at the follow-up visit for measurement of plasma glucose and lipid concentrations using standard techniques. In the Botnia study, blood samples were drawn at –10, 0, 30, 60 and 120 min of the OGTT. Insulin sensitivity index (ISI) from the OGTT was calculated as $10{,}000/\sqrt{((\text{fasting plasma glucose} \times \text{fasting plasma insulin})(\text{mean OGTT}_{\text{glucose}} \times \text{mean OGTT}_{\text{insulin}}))}$[26]. The basal insulin resistance index (HOMA) was calculated from fasting insulin and glucose concentrations (see URLs section below). β-cell function was assessed as corrected incremental insulin response during OGTT (CIR = (100 × insulin at 30 min or 40 min in MPP))/((glucose at 30 min or 40 min in MPP) × (glucose 30 min or 40 min in MPP – 3.89))[27] or as disposition index, that is, insulin secretion adjusted for insulin sensitivity (CIR × ISI).

Plasma glucose was measured by hexokinase (MPP, FUSION), glucose oxidase (Botnia, FUSION, METSIM) methods. Plasma insulin concentrations were measured by an ELISA assay (Dako, Cambridgeshire; Botnia study), by a local radioimmunoassay (MPP), by radioimmunoassay using dextran-charcoal separation (FUSION) or by a commercial double-antibody solid-phase radioimmunoassay (METSIM).

Genotyping. In the DGI and FUSION GWAS, genotyping was done using Affymetrix 500K chip array[1] and Illumina HumanHap300 BeadChip Version 1.0 (ref. 2). In the FUSION and METSIM studies, SNP rs10830963 was genotyped by Sequenom iPlex gold SBE (Sequenom); in all other replication studies rs10830963 was genotyped by an allelic discrimination assay-by-design method on ABI 7900 (Applied Biosystems). Genotypes were in Hardy-Weinberg equilibrium. In MPP and Botnia, we obtained an average genotyping success rate of >95% and the concordance rate was 98.7%, using two different methods (allelic discrimination on ABI7900 and Affymetrix). Replication genotyping for FUSION and METSIM studies was done using Sequenom iPlex gold SBE (Sequenom).

Immunocytochemistry. For histochemical analysis pancreatic specimens were dissected, fixed overnight in Stefanini's solution (2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffered saline, pH 7.2), rinsed thoroughly in Tyrode solution containing 10% sucrose and frozen on dry ice. Sections (10 mm thickness) were cut and thaw-mounted on slides. Antibodies were diluted in PBS (pH 7.2) containing 0.25% BSA and 0.25% Triton X-100. Sections were incubated with primary antibodies (goat antibody to melatonin receptor 1B (code sc-13177; dilution 1:400, Santa Cruz Biotechnology)); goat antibody to melatonin receptor 1A (code sc-13186, dilution 1:400, Santa Cruz Biotechnology) and guinea pig antibody to proinsulin (code 9003; dilution 1:2,560; EuroDiagnostica) overnight at 4 °C in moisturizing chambers. The sections were rinsed in PBS with Triton X-100 for 2 × 10 min. Thereafter secondary antibodies with specificity for goat or guinea pig IgG, and coupled to either fluorescein isothiocyanate (FITC) or Texas-Red (Jackson), were applied on the sections. Incubation was for 1h at room temperature in moisturizing chambers. The sections were again rinsed in PBS with Triton X-100 for 2 × 10 min and then mounted in PBS:glycerol, 1:1. The specificity of immunostaining was tested using primary antisera pre-absorbed with homologous antigen (100 μg of peptide per ml antiserum at working dilution). Immunofluorescence was examined in an epifluorescence microscope (Olympus, BX60). By changing filters the location of the different secondary antibodies in double staining was determined. Images were captured with a digital camera (Nikon DS-2Mv)[28].

Gene expression using real-time PCR. Total RNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen) at the Human Tissue Facility of Lund University Diabetes Center (LUDC); or by RNeasy protect mini kit (Qiagen) as previously described[15] at the Joslin Islet Cell Resource Center (Joslin); or by Trizol (Invitrogen) and further purification using RNeasy mini kit (Qiagen) at the National Human Genome Research Institute (NHGR). RNA quantity was determined by evaluating the absorbance at 260 and 280 nm in a Perkin-Elmer spectrophotometer (Waltham), and quality was assessed by running samples on Agilent 2100 Bioanalyzer (Agilent Technologies) at Joslin. cDNA was synthesized from 0.4 μg total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences) (at LUDC); 0.5 μg total RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) (at NHGR); and 1 μg total RNA using iScript cDNA synthesis kit (Biorad) (at Joslin). TaqMan gene expression assays were purchased from Applied Biosystems for the various target genes: Hs00173794_m1 directed against human *MTNR1B* and *HPRT* (hypoxanthine-guanine phosphoribosyl transferase) (at LUDC and NHGR) and *PPIA* (cyclophilin) (at Joslin), which served as endogenous control gene. Q-PCR reactions were done on the ABI 7900HT (Applied Biosystems) at LUDC and NHGR by mixing 2× TaqMan Universal Master Mix, 20× TaqMan Gene Expression Assays, nuclease-free water and cDNA for a final reaction volume of 10 μl (at LUDC), as described earlier[29] (at Joslin). The relative quantity of *MTNR1B*

mRNA was calculated using the comparative threshold method (Ct-method) (at LUDC and NHGR). All experiments were performed in triplicate.

For microarray experiments, 100 ng total RNA was subjected to two rounds of amplification (GeneChip Two-Cycle Kit, Affymetrix), and biotinylated RNA was generated using GeneChip IVT Labeling Kit (Affymetrix). RNA products were fragmented and hybridized to GeneChip Human HG U 133A Array (Affymetrix). The array data were normalized and analyzed using DNA-Chip Analyzer (dChip) software (see URLs section below, last accessed in January 2008) that assesses the standard errors for the expression indexes and calculates confidence intervals for fold changes (Joslin, NHGR).

Effect of melatonin on insulin secretion. To determine the effects of melatonin on insulin secretion, we incubated the clonal β cells from the line 832/13 with 0.1 μM melatonin for 1 h. Then, the amount of released insulin into the buffer was determined by radioimmunoassay.

Statistical analyses. Differences in expression levels were tested by analysis of variance or nonparametric Mann-Whitney tests. The odds ratios for risk of developing T2D were calculated using logistic regression analyses adjusted for age at participation and time to last follow-up, body mass index and sex. Multivariate linear regression analyses were used to test genotype–phenotype correlations adjusted for age, sex, body mass index (apart from body mass index phenotype) and for within-family dependence. Non-normally distributed variables were log-transformed before analysis. Analysis of FUSION FSIGT and METSIM OGTT data was carried out using a regression framework in which regression coefficients were estimated in the context of a variance component model to account for relatedness among individuals[30]. Trait values for both studies were adjusted for age and age squared. For FUSION data sex was included as an additional covariate. Analyses were carried out in nondiabetic individuals excluding those known to be taking medications that directly affect glucose or insulin concentrations. Covariate-adjusted trait values were transformed to approximate univariate normality by applying an inverse normal scores transformation; the scores were ranked, ranks were transformed into quantiles and quantiles were converted to normal deviates.

All statistical analyses were performed using SPSS version 14.0, PLINK, Stata (StataCorp) or MERLIN[30].

URLs. Diabetes Trial Unit, http://www.dtu.ox.ac.uk/, dChip software, http://biosun1.harvard.edu/complab/dchip/; PLINK, http://pngu.mgh.harvard.edu/~purcell/plink/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

V.L.: DGI GWAS, data analysis and draft of the report. C.L.F.N., M.R.E.: *in vitro* expression experiments and analysis, and draft of the report. N.W.: immunocytochemistry. A.J.: genotyping and data analysis. P.S.: *in vitro* expression experiments. M. Bugliani: microarray and human islets experiments. R.S.: DGI GWAS analysis. M.F.: *in vitro* physiology. N.P.: genotyping. B.I., T.T.: phenotyping in the Botnia study. P.N.: phenotyping in the Malmoe study. J.K.: data analysis in METSIM study. J.T.: phenotyping in the FUSION study. M. Boehnke: PI of the FUSION study. D.A.: PI of the DGI study. F.S.: immunocytochemistry. J.G.E.: phenotyping in the Helsinki Birth Cohort Study. A.U.J.: FUSION GWAS and data analysis. M.L.: PI of the METSIM study. P.M.: microarray and human islets experiments. R.M.W.: FUSION GWAS analysis. H.M.: design and supervision of *in vitro* study experiments and draft of the report. L.G. designed and supervised all parts of the study and drafted the report. All researchers took part in the revision of the report and approved the final version.

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