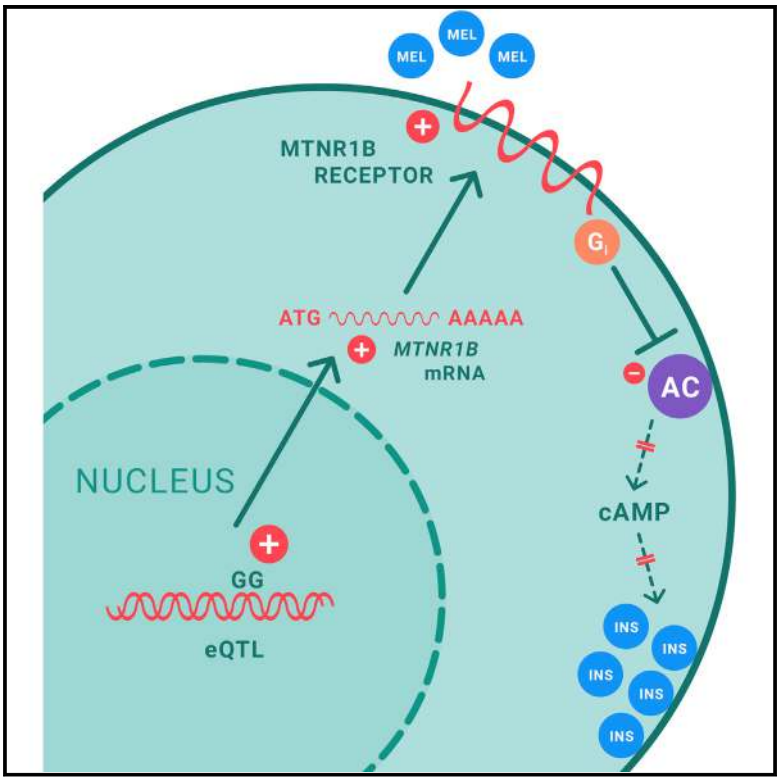
Clinical and Translational Report

Cell Metabolism

Increased Melatonin Signaling Is a Risk Factor for Type 2 Diabetes

Graphical Abstract



Highlights

- rs10830963 is an eQTL in human islets conferring increased *MTNR1B* mRNA expression
- Melatonin inhibits cAMP rises in mouse islets and clonal insulin-secreting cells
- Melatonin blocks insulin release in mouse islets and clonal insulin-secreting cells
- Melatonin's inhibition of insulin release is stronger in risk allele carriers

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In Brief

Tuomi et al. show that a common (about 30%) human type 2 diabetes risk variant of the melatonin receptor 1B gene affects insulin release. A recall-by-genotype study demonstrated that melatonin treatment inhibits insulin secretion, with at-risk carriers exhibiting higher glucose levels. Melatonin might have a protective role in preventing nocturnal hypoglycemia.

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Increased Melatonin Signaling Is a Risk Factor for Type 2 Diabetes

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SUMMARY

Type 2 diabetes (T2D) is a global pandemic. Genome-wide association studies (GWASs) have identified >100 genetic variants associated with the disease, including a common variant in the melatonin receptor 1 b gene (*MTNR1B*). Here, we demonstrate increased *MTNR1B* expression in human islets from risk G-allele carriers, which likely leads to a reduction in insulin release, increasing T2D risk. Accordingly, in insulin-secreting cells, melatonin reduced cAMP levels, and *MTNR1B* overexpression exaggerated the inhibition of insulin release exerted by melatonin. Conversely, mice with a disruption of the receptor secreted more insulin. Melatonin treatment in a human recall-by-genotype study reduced insulin secretion and raised glucose levels more extensively in risk G-allele carriers. Thus, our data support a model where enhanced melatonin signaling in islets reduces insulin secretion, leading to hyperglycemia and greater future risk of T2D. The findings also imply that melatonin physiologically serves to inhibit nocturnal insulin release.

INTRODUCTION

Type 2 diabetes (T2D) is a global disorder rapidly increasing in prevalence. It is projected to afflict 350 million people in 2030 (Shaw et al., 2010). The burden on society as well as the afflicted individual is tremendous. The disease is of multifactorial origin: environmental factors, associated with a modern lifestyle (obesity, physical inactivity, increased age), trigger the disease in genetically susceptible individuals (Prasad and Groop, 2015). Genome-wide association studies (GWASs) have identified >100 genetic variants associated with the disease (Prasad and Groop, 2015; Visscher et al., 2012), including a common variant (~30% of the population) in the melatonin receptor 1 b gene (*MTNR1B*) (Bouatia-Naji et al., 2009; Lyssenko et al., 2009; Prokopenko et al., 2009). The mechanisms, however, by which melatonin affects glucose metabolism and the development of T2D remain largely unknown. Notably, the SNP rs10830963 in the *MTNR1B* gene showed a stronger association with glucose levels in nondiabetic individuals than with risk of future T2D (Lyssenko et al., 2009). Although the majority of the identified genes points to failure of pancreatic β cells to release insulin as being the main culprit in the pathogenesis of the disease, the underlying molecular mechanisms have remained largely unresolved for most genes identified by GWAS (Torres et al., 2013). Moreover, information about these genetic risk variants has not yet been translated into the clinical setting, where improved preventive and diagnostic tools are urgently needed (Lyssenko et al., 2008). It is becoming increasingly clear that treatment of metabolic diseases is not equally efficacious in all individuals (Anderson et al., 2012). One means of meeting these challenges is individualized treatment. Here, improved understanding of pathogenetic processes is required to allow tailored treatment on an individual basis. Understanding the impact of genotype, which reflects or even underlies such processes, is one way to reach this

goal, but studies proving this concept are only now starting to emerge (Tang et al., 2014).

Melatonin is a hormone produced predominantly in the pineal gland (Mulder et al., 2009). Its release is triggered by loss of light exposure to the retina. Hence, melatonin indicates the time of day, or ambient light, to various organs and tissues in the body—it is hereby a "Zeitgeber," entraining circadian rhythm. Indeed, control of circadian rhythm at several levels, including the pancreatic β cell (Marcheva et al., 2010), has been implicated in normal metabolic control, as well as the development of T2D (Marcheva et al., 2009; Peschke et al., 2015). The hormone signals at the cellular level through two receptors: 1A and 1B (MT1 and MT2 in the mouse) (von Gall et al., 2002). Both receptors mainly act via interfering with the formation of cAMP through inhibitory G proteins (G_i), but also other signaling pathways are employed (Mulder et al., 2009; von Gall et al., 2002). The pleiotropism at the level of both receptor and second messenger probably explains why the reported effects on insulin release have not yielded a clear understanding of the regulatory role of melatonin in insulin release (Mulder et al., 2009). Thus, both inhibitory and stimulatory effects of melatonin on insulin secretion have been reported (Kemp et al., 2002; Peschke et al., 2006).

Against this background, it is intriguing that we and others have found that a variant of the *MTNR1B* gene is associated with elevated plasma glucose levels, a reduction of the early insulin response to both oral and intravenous glucose, a faster deterioration of insulin secretion over time, and increased future risk of T2D (Bouatia-Naji et al., 2009; Lyssenko et al., 2009; Prokopenko et al., 2009). This association has subsequently been confirmed in other populations (Jonsson et al., 2013; Renström et al., 2015; Rönn et al., 2009). Despite the very robust genetic association, a molecular understanding of why melatonin signaling is involved in the pathogenesis of T2D has still not been reached. To resolve this issue, we performed experimental studies in human islets, INS-1 832/13 β cells, and mice, as well as clinical studies in humans. We show that the rs10830963 risk variant of *MTNR1B* is an expression quantitative trait locus (eQTL) conferring increased expression of *MTNR1B* mRNA in human islets. Experiments in INS-1 832/13 β cells and *Mt2* knockout mice ($Mt2^{-/-}$) establish that melatonin signaling results in inhibition of insulin release. Translation to humans in a recall-by-genotype study demonstrates that melatonin treatment inhibits insulin secretion in all subjects, but carriers of the risk variant are more sensitive to this inhibitory effect of melatonin. Together, these observations support a model in which a genetically determined increase in melatonin signaling underlies impaired insulin secretion, a pathogenetic hallmark of T2D.

RESULTS AND DISCUSSION

rs10830963 Is an eQTL for *MTNR1B* in Human Islets

We have previously reported that mRNA levels of *MTNR1B* are increased in isolated islets from a small group of older donors (n = 25; age >45 years) carrying the rs10830963 risk variant (Lyssenko et al., 2009). To establish that the risk allele is indeed an eQTL, we quantified *MTNR1B* mRNA in relation to the rs10830963 SNP in islets from 204 donors of Scandinavian descent, using RNA sequencing. Carriers of one or two risk alleles exhibited a 2- and 4-fold increase in *MTNR1B* mRNA expression compared with carriers of two copies of the non-risk allele (Figure 1A). Expression of *MTNR1A* was not affected by either of these genotypes. The rs10830963/*MTNR1B* eQTL has not been reported in brain, which exhibits the highest level of *MTNR1B* expression, nor in any other tissue, and thus seems to be specific for human pancreatic islets. Accumulating data thereby establish *MTNR1B* as one of the strongest eQTLs in human islets (Fadista et al., 2014; Lyssenko et al., 2009).

A recent study suggests a plausible molecular mechanism for the upregulation of *MTNR1B* in islets in risk allele carriers (Gaulton et al., 2015). FOXA2 binding motifs found by chromatin immunoprecipitation sequencing (ChIP-seq), and used to identify causal genetic variants for susceptibility to T2D, are enriched at the *MTNR1B* locus. This is thought to enable binding of FOXA2 and/or other transcription factors. Subsequent studies revealed that rs10830963 preferentially binds the transcription factor NEUROD1 in EndoC-βH1 cells, a cell line derived from fetal human β cells (Andersson et al., 2015; Ravassard et al., 2011). In fact, the *MTNR1B* risk G-allele creates a NEUROD1 consensus binding site. Accordingly, the region surrounding rs10830963, and comprising the G-allele, shows increased enhancer activity in EndoC-βH1 cells. Whether this mechanism mediates increased expression of *MTNR1B* also in primary islet cells remains to be shown.

The effects of melatonin are mediated by two homologous, but distinct, receptors, which are expressed in a cell-specific fashion in mice and humans (Nagorny et al., 2011; Ramracheya et al., 2008; von Gall et al., 2002). Here, we focused on *MTNR1B*/*Mt2* because it harbors the rs10830963 risk variant, mapping to its single 11.5 kb intron. However, some data suggest that MT1 is the predominant receptor for melatonin signaling under normal conditions in INS-1 832/13 β cells and mouse islets (Mühlbauer et al., 2012). In mouse, we have demonstrated that MT1 receptors are mainly expressed in the glucagon-producing α cell (Nagorny et al., 2011), whereas MT2 receptors are mainly found in β cells (Lyssenko et al., 2009; Nagorny et al., 2011). Indeed, these results confirmed a previous study, which identified *MTNR1A* mRNA in human α cells (Ramracheya et al., 2008). Here, it was further found that melatonin increases glucagon secretion from dispersed human islet cells, and potentially insulin secretion via an indirect effect mediated by glucagon on the β cell (Ramracheya et al., 2008). Very recently, RNA sequencing of purified adult human islet cells revealed that *MTNR1B* is expressed several-fold higher than *MTNR1A* both in α and β cells; expression of *MTNR1B* is similar in α and β cells (Blodgett et al., 2015).

Increased Expression of *MTNR1B* in Insulin-Secreting INS-1 832/13 β Cells

Having confirmed that the rs10830963 *MTNR1B* risk variant conferred increased expression of receptor mRNA in human islets, we continued to investigate whether this results in a gain of function. To this end, we overexpressed the receptor in clonal insulin-secreting cells (INS-1 832/13), using a recombinant adenovirus driving expression of *MTNR1B* by the rat insulin promoter 2 (*Rip2*). Given that *MTNR1B*/*Mt2* expression constitutively is low in β cells, Adv-*Rip2*-*MTNR1B* conferred a robust increase in receptor mRNA (see Figure S1A available online). Insulin release in response to 16.7 mM glucose during a 1 hr incubation of these

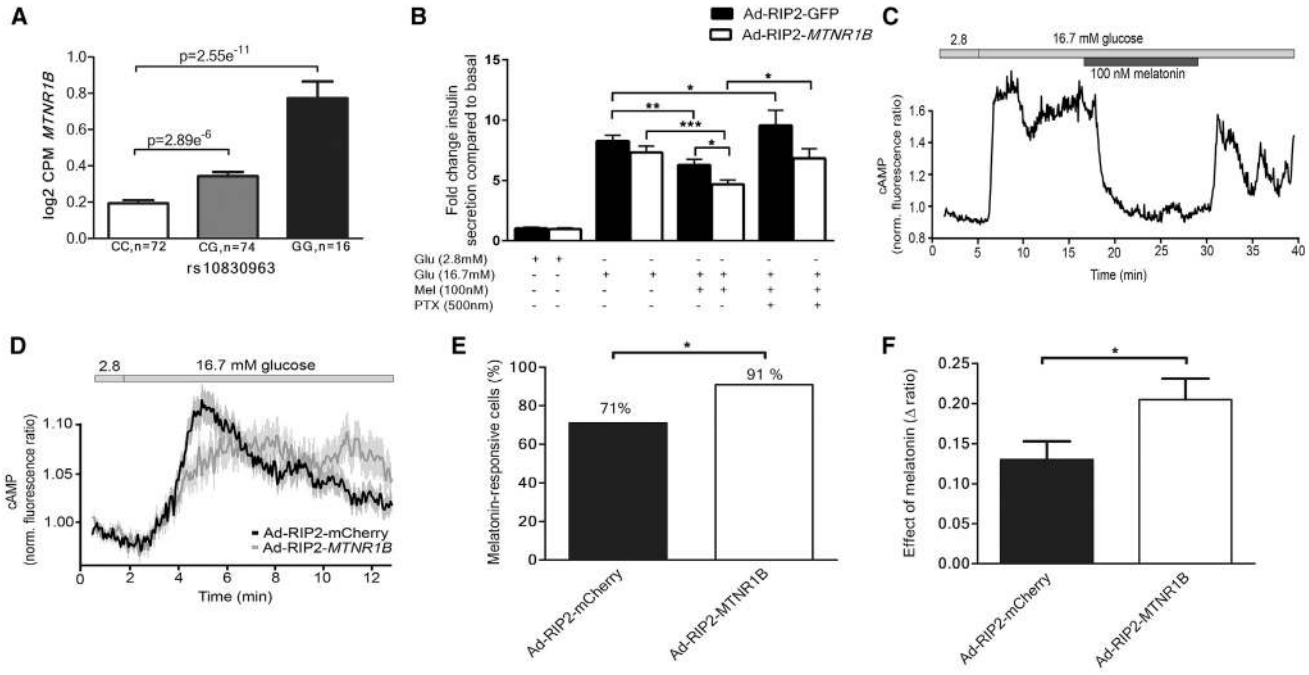

Figure 1. *MTNR1B* Overexpression, Found in Human Islets, Increases the Melatonin-Mediated Inhibition of Insulin Secretion in INS-1 832/13 β Cells via a Decrease in cAMP Levels

(A) RNA sequencing of islets from 204 human donors shows that *MTNR1B* mRNA expression is increased in carriers of the G-risk allele. Comparisons between genotypes were made by Student's t test. Nominal p values are given; mean + SEM.

(B) Melatonin inhibits insulin in *MTNR1B*-overexpressing INS-1 832/13 β cells. The inhibitory effect of melatonin on insulin was reversed by prior incubation with pertussis toxin (PTX). Wilcoxon matched-pairs signed rank test: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Mean ± SEM. Ad-*Rip2-MTNR1B*, adenovirus expressing *MTNR1B* under control of the rat insulin 2 promoter (*Rip2*); Ad-*Rip2-GFP*, control adenovirus expressing GFP under control of *Rip2*.

(C) Melatonin lowers cAMP in INS-1 832/13 β cells. Recording of intracellular cAMP in a single INS-1 832/13 β cell during exposure to glucose and melatonin. Representative of 55 cells from four experiments.

(D) Online recordings of intracellular cAMP in INS-1 832/13 β cells transduced with Ad-*Rip2*-mCherry (control) or Ad-*Rip2-MTNR1B* and stimulated with an increase of the glucose concentration. Means ± SEM of the cAMP reporter fluorescence ratio for 139 and 129 cells, respectively, for the two groups. The initial glucose response tended to be lower, but the later response increased in the *MTNR1B*-overexpressing cells compared to control.

(E) Percentage of INS-1 832/13 β cells responding to melatonin after transduction with Ad-*Rip2*-mCherry (n = 38 cells) compared to Ad-*Rip2-MTNR1B* (n = 33 cells). *$p < 0.05$ (chi-square test).

(F) Magnitude of the cAMP-lowering effect of melatonin. Means ± SEM for the change in cAMP reporter fluorescence ratio induced by 100 nM melatonin in the glucose-responsive INS-1 832/13 β cells. n = 27 cells for Ad-*Rip2*-mCherry and 30 cells for Ad-*Rip2-MTNR1B*. *$p < 0.05$ (data normally distributed; Student's t test). The accentuated response in *MTNR1B*-expressing cells is likely due to a higher steady-state cAMP level in these INS-1 832/13 β cells.

See also Figure S1.

cells was unaffected by *MTNR1B* overexpression (Figure 1B). However, addition of 100 nM melatonin reduced insulin release from *MTNR1B*-overexpressing cells more extensively than from control cells.

The melatonin receptors inhibit adenylate cyclase activity via a pertussis toxin (PTX)-sensitive G_i protein, with a consequent decrease in cAMP production (Mulder et al., 2009). Therefore, the effect of melatonin on intracellular cAMP levels was investigated in single insulin-secreting cells expressing a fluorescent cAMP reporter (Dyachok et al., 2006). An increase in the glucose concentration from 2.8 to 16.7 mM robustly induced a rise of cAMP, which was reversibly suppressed by melatonin (Figure 1C). Moreover, *MTNR1B* overexpression slightly, but not significantly, blunted the initial glucose response (Figure 1D) but significantly increased the fraction of melatonin-responsive cells and effect of the hormone (Figures 1E and 1F). Conversely, addition of PTX, blocking signaling from the MTNR1B receptor, prevented the inhibitory effect of melatonin on insulin release in control as well as in *MTNR1B*-overexpressing cells (Figure 1B). Thus, melatonin inhibited insulin release via reduced formation of cAMP, and this was accentuated by *MTNR1B* overexpression.

In Vivo Glucose Metabolism in Melatonin Receptor 1B/*Mt2* Knockout Mice

To further understand the role of melatonin signaling for metabolic control, we examined glucose metabolism in mice with a genetic ablation of *Mt2*, the murine equivalent of *MTNR1B* in humans (von Gall et al., 2002). *Mt2*$^{-/-}$ mice fed normal chow diet gradually became heavier than wild-type (WT) control mice (Figures S2A and S2B). An intravenous glucose tolerance test (IVGTT; Figures 2A and 2B; Figures S2C–S2E) revealed increased insulin secretion during the challenge. This exaggerated response to glucose was more robust in female than in male mice (Figures S2F–S2H). Despite elevated insulin levels, plasma glucose levels and glucose elimination rates (Kg) in *Mt2*$^{-/-}$ mice remained unchanged (Figure 2C).

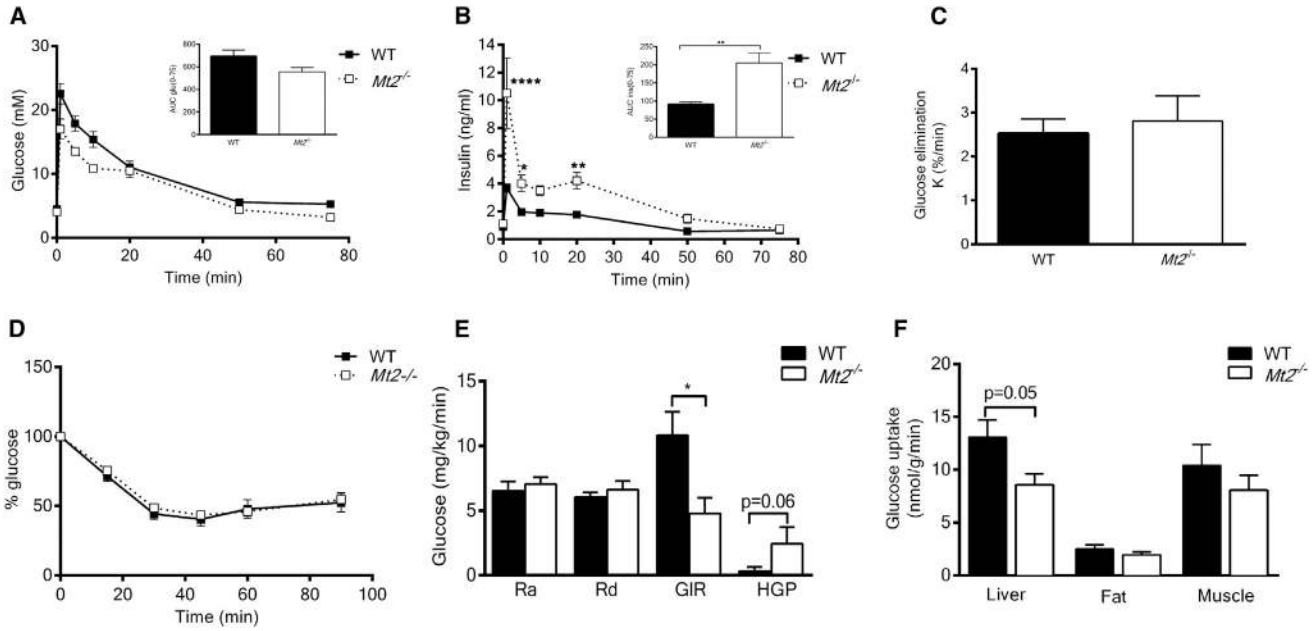

Figure 2. *Mt2*$^{-/-}$ Mice Exhibit Increased Insulin Release and Reduced Hepatic Insulin Sensitivity

(A) Intravenous glucose tolerance test (IVGTT) in 12-week-old female WT (black; n = 12) and *Mt2*$^{-/-}$ (white; n = 4) mice. Inset is area under curve (AUC). Mean ± SEM.

(B) Plasma insulin levels in 12-week-old female WT (black; n = 12) and *Mt2*$^{-/-}$ (white; n = 4) mice during IVGTT in (A). Inset is AUC. Mean ± SEM. Mann-Whitney U-test for AUC; **p < 0.01. Two-way ANOVA with Bonferroni's multiple comparisons post hoc was used for testing statistical significance between genotypes at different time points. *p < 0.05, **p < 0.01, and ****p < 0.0001. Mean ± SEM.

(C) Glucose elimination rate (K) in 12-week-old WT (black; n = 12) and *Mt2*$^{-/-}$ (white; n = 4) female mice during IVGTT in (A). Glucose elimination rate (K) in %/min calculated from linear regression over the first 10 min after glucose injection. Mean ± SEM.

(D) Insulin tolerance test (ITT) in 24-week-old WT (black; n = 5) and *Mt2*$^{-/-}$ (white; n = 15) female mice. Glucose values expressed as % glucose, where glucose at 0 min is 100%. Mean ± SEM.

(E) Hyperinsulinemic-euglycaemic clamp in 26-week-old WT (black; n = 8) and *Mt2*$^{-/-}$ (white; n = 6) female mice. Ra = Rate of appearance of 3-^{3}H- glucose during basal period = whole-body glucose turnover = endogenous hepatic glucose production (HGP); Rd = rate of disappearance of 3-^{3}H- glucose (whole-body glucose turnover) during clamp period; GIR = glucose infusion rate (20% glucose); Rd during clamp = GIR + HGP during clamp. Mann-Whitney U-test: *p < 0.05. Mean ± SEM.

(F) Insulin-stimulated glucose uptake in liver, fat, and muscle during steady state of hyperinsulinemic-euglycaemic clamp in (E). Mann-Whitney U-test: p = 0.05. Mean ± SEM.

See also Figures S2 and S3.

Increased levels of circulating insulin in the face of unaltered plasma glucose levels imply decreased systemic insulin sensitivity, but insulin tolerance was similar in *Mt2*$^{-/-}$ and WT mice (Figure 2D). To further dissect glucose homeostasis in *Mt2*$^{-/-}$ mice, we performed hyperinsulinemic-euglycaemic clamps. While neither basal whole-body glucose turnover (Ra) nor clamp whole-body glucose turnover (Rd) differed between the strains, the glucose infusion rate (GIR) was significantly lower in *Mt2*$^{-/-}$ mice during the clamp (Figure 2E). The hepatic glucose production (HGP) during the clamp was virtually abolished in WT mice, while the *Mt2*$^{-/-}$ mice failed to inhibit endogenous hepatic glucose production to the same extent (Figure 2E; Figure S3A). Since the rate of glucose uptake is the sum of GIR and residual HGP during the clamp, impaired suppression of hepatic glucose output most likely explained the normal Rd in *Mt2*$^{-/-}$ mice.

Together with moderately reduced glucose uptake in liver (Figure 2F), these findings suggest impaired insulin sensitivity at the level of the liver. Glucose uptake in muscle trended toward a decrease, while that in fat remained unaltered (Figure 2F). Collectively, these data suggest that the liver of *Mt2*$^{-/-}$ mice accounted for the reduction in insulin sensitivity. Increased hepatic glucose production in *Mt2*$^{-/-}$ mice is presumably an adaptive change, which evolved to maintain euglycaemia in the face of enhanced insulin release. However, we could not find any clear explanation for increased hepatic glucose production in *Mt2*$^{-/-}$ mice since glycogen synthesis and glycolytic rate remained unchanged in liver, fat, and skeletal muscle (Figures S3B and S3C).

With respect to basal whole-body metabolism in *Mt2*$^{-/-}$ mice, our data agree with those published previously, i.e., no differences in circulating plasma glucose or insulin levels (Bazwinsky-Wutschke et al., 2014). To the best of our knowledge, no glucose tolerance tests or clamp studies in melatonin receptor knockout mice have been reported.

β Cell Mass, Insulin Secretion, and cAMP in Melatonin Receptor 1B/*Mt2* Knockout Mice

To further clarify why insulin secretion in vivo is exaggerated in the *Mt2*$^{-/-}$ mice, we examined β cell mass. *Mt2*$^{-/-}$ mice

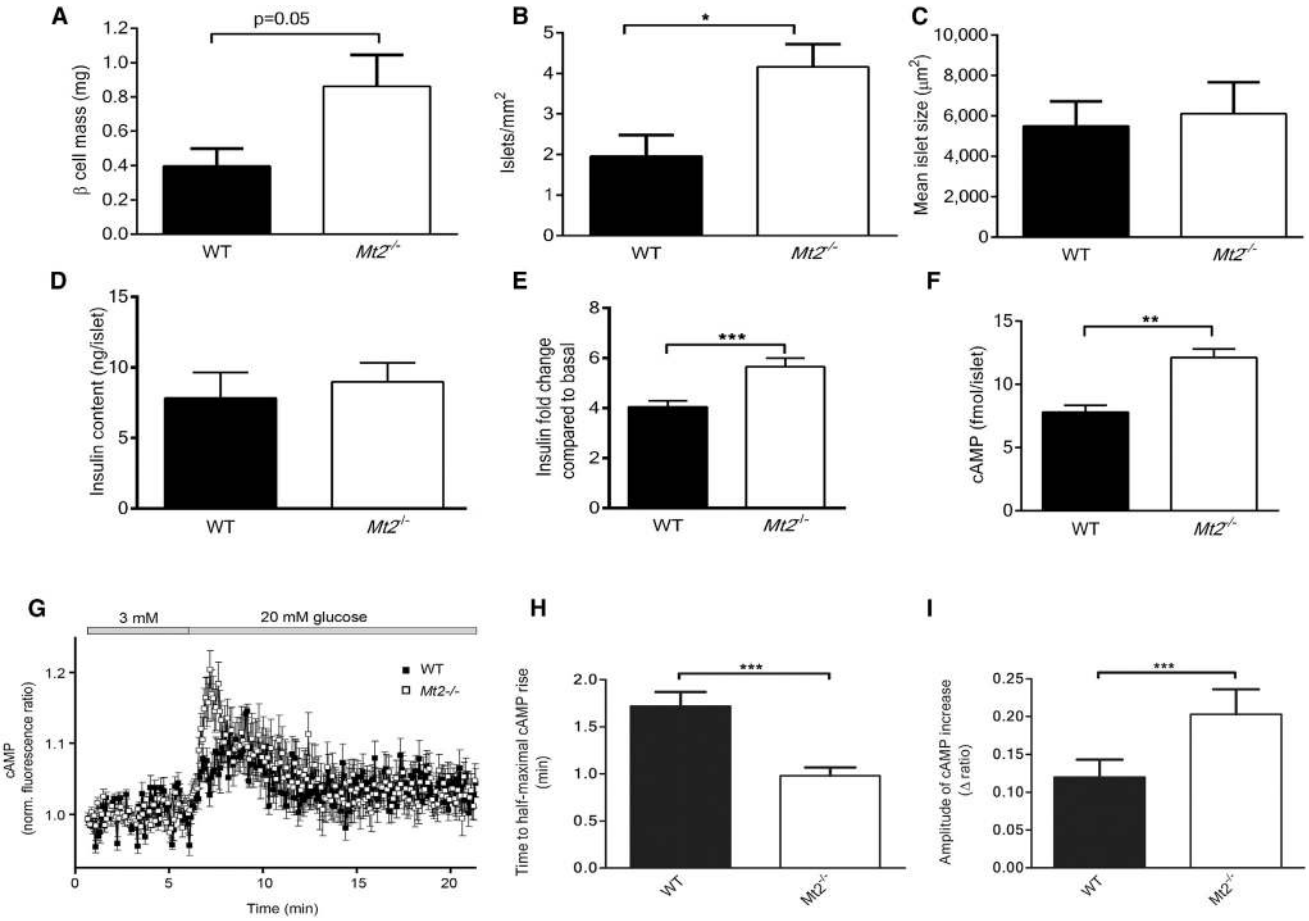

Figure 3. *Mt2*$^{-/-}$ Mice Exhibit Increased Insulin Release Due to Increased β Cell Mass and Exaggerated cAMP Responses

(A) β cell mass in 16- to 19-week-old male and female WT (black; n = 6) and *Mt2*$^{-/-}$ (white; n = 10) mice. β cell mass is calculated as islets number/total area × pancreas weight. Mann-Whitney U-test: p = 0.05; mean ± SEM.

(B) Number of islets/mm^2 area calculated as islets/total pancreatic area in 16- to 19-week-old WT (black; n = 6) and *Mt2*$^{-/-}$ (white; n = 10) male and female mice. Mean ± SEM. Mann-Whitney U-test: *p < 0.05; mean ± SEM.

(C) Mean islet size in 16- to 19-week-old WT (black; n = 6) and *Mt2*$^{-/-}$ (white; n = 10) male and female mice.

(D) Islet insulin content of 12-week-old WT (black; n = 7) and *Mt2*$^{-/-}$ (white; n = 9) female mice. Mean ± SEM.

(E) Fold change (FC) of insulin secretion over basal (1 mM glucose) from islets of 12-week-old WT (black; n = 7) and *Mt2*$^{-/-}$ (white; n = 9) female mice after 1 hr batch incubation with 1 or 15 mM glucose. Mann-Whitney U test: ***p < 0.001. Mean ± SEM.

(F) Basal cAMP levels in islets of 14-week-old WT (black; n = 3) and *Mt2*$^{-/-}$ (white; n = 3) female mice. Islets were cultured overnight in RPMI media containing 5.5 mM glucose. Mann-Whitney U test: **p < 0.01. Mean ± SEM.

(G) Online recordings of intracellular cAMP in cells within intact islets from WT and *Mt2*$^{-/-}$ mice during glucose stimulation. Means ± SEM for 15 and 22 cells in islets from two (WT) and three (*Mt2*$^{-/-}$) independent isolations, respectively.

(H) Time to half-maximal increase of cAMP in islet cells stimulated with an increase of the glucose concentration from 3 to 20 mM. Means ± SEM for 15 cells from WT (two isolations) and 22 cells from *Mt2*$^{-/-}$ mice (three isolations). ***p < 0.001 for difference (two-tailed Mann-Whitney U test).

(I) Amplitude of maximal glucose-induced cAMP increase in islet cells from WT and *Mt2*$^{-/-}$ mice. Means ± SEM *p < 0.05 for difference (two-tailed Mann-Whitney U test).

displayed greater β cell mass compared to WT mice (Figure 3A). The increased β cell mass was due to an increase in number of islets but not in mean islet size (Figures 3B and 3C). Accordingly, insulin content was similar in *Mt2*$^{-/-}$ and WT islets (Figure 3D).

Next, we examined insulin secretion from *Mt2*$^{-/-}$ islets. Here, *Mt2*$^{-/-}$ islets displayed an accentuated secretory response compared with WT islets: in *Mt2*$^{-/-}$ islets, stimulated secretion at 16.7 mM glucose increased 6-fold over basal versus 4-fold in WT islets (Figure 3E). This could be explained by increased levels of cAMP, mediated by the loss of melatonin signaling, which normally would lower the second messenger level in β cells. Indeed, we observed higher levels of basal cAMP in *Mt2*$^{-/-}$ islets (Figure 3F). Moreover, online recordings with the fluorescent cAMP reporter revealed a more pronounced initial glucose-induced cAMP elevation in islet cells from *Mt2*$^{-/-}$ mice (Figure 3G). A closer analysis of these data in *Mt2*$^{-/-}$ islets showed that the time to half-maximal rise in cAMP level was significantly decreased and the amplitude of the cAMP elevation increased (Figures 3H and 3I). Together, these data show that insulin secretion in *Mt2*$^{-/-}$ islets was enhanced. This can be

explained by an increased secretory response and greater β cell mass. The former can be attributed to raised cAMP levels due to the lost inhibitory effect of melatonin (Mulder et al., 2009).

Data from us and others thus suggest that MTNR1B signaling serves to inhibit insulin secretion and, hereby, influences glycaemic control. Here, this is based on in vitro and in vivo studies in cells, mice, and humans: overexpression of *MTNR1B* in insulin-secreting cells accentuated a reduction in insulin release in the presence of melatonin, and, conversely, inactivation of the receptor in mice resulted in enhanced insulin secretion, i.e., the reverse phenotype, or loss of function (mice) as opposed to gain of function (β cells and humans). The receptor most likely mediated its effect via a reduction of cAMP, an important potentiator of insulin secretion (Mulder et al., 2009). This is based on the notion that during single-cell, real-time recordings of cAMP, melatonin reduced cAMP in β cells and that PTX (G_i protein-inhibitor) reversed the inhibitory effect of melatonin on insulin release in *MTNR1B*-overexpressing cells and, finally, that basal cAMP levels were raised in islets from $Mt2^{-/-}$ mice.

Knockout mice for melatonin receptors have previously been studied with mixed results. Isolated islets from $Mt1^{-/-}$ mice as well as double knockout mice for *Mt1* and *Mt 2* lose the inhibitory effect of melatonin on insulin secretion (Mühlbauer et al., 2012). In $Mt2^{-/-}$ mice, there is a 40% nonsignificant reduction in insulin release upon addition of melatonin. If there would be an inhibitory effect of melatonin on insulin secretion in $Mt2^{-/-}$ mice, it could be mediated via remaining Mt1 receptors in islets. However, these results are hard to interpret because the authors studied 6 hr incubations of islets at 8.6 mM in full medium containing other potential secretagogues. Therefore, it may be difficult to distinguish between an acute and a more chronic effect of melatonin on islets and whether stimulation is specific for any given fuel.

The specific impact of melatonin signaling on β cell function has previously been studied in INS-1 cells stably transfected with *MTNR1B* (Mühlbauer et al., 2011). This manipulation led to dose-dependent exaggeration of an inhibitory effect of melatonin on insulin secretion, which is PTX sensitive, and mediated by decreases in whole-cell cAMP. Interpretation of the results is, however, confounded by the fact that only one clone has been studied, that data represent a chronic situation where signaling from MTNR1B is exaggerated, and that incubations of cells have largely been for 6 hr. Here, we have created depth to the understanding by using a single-cell approach in both clonal and primary cells. Imaging of the responses in cellular cAMP levels have mapped out how β cells and islets respond to melatonin in terms of cAMP.

Recall-by-Genotype of Carriers of the *MTNR1B* Risk Variant

If the trait conferred by rs10830963 is indeed a gain of function, as suggested by our in vitro and mouse in vivo data, then activation of the system in vivo in humans would lead to a reduction of glucose-stimulated insulin secretion. To test this hypothesis, 23 nondiabetic individuals with two copies of the risk allele (GG) and 22 carriers of two copies of the nonrisk allele (CC) were administered 4 mg of melatonin at bedtime for 3 months. There were no differences in age, gender, body mass index, or family history of diabetes (see "Melatonin Intervention Trial" in Supplemental Information). During an oral glucose tolerance test (OGTT), preceding administration of melatonin, glucose concentrations were higher and the first-phase insulin response lower in GG versus CC genotype carriers (Figures 4A and 4C; Table 1), confirming our previous results (Lyssenko et al., 2009). Insulin secretion adjusted for insulin sensitivity (disposition index; DI) was three times higher in CC than in GG carriers (Table 1).

After 3 months of melatonin treatment, there was a clear decrease in first-phase insulin release and an increase in glucose concentrations in all subjects (Figure S4; Table S1). This was particularly clear in GG carriers (Figures 4B and 4D; Table 1), despite identical dosage of melatonin and similar plasma melatonin concentrations in GG and CC carriers. When compared with baseline data, measures of insulin secretion were lower in the GG carriers after melatonin treatment (Figures 4E and 4F; Table 1), particularly the corrected insulin response (CIR) during the first 30 min of the OGTT ($p = 0.008$; Figure 4E). Notably, insulin sensitivity increased after melatonin treatment regardless of genotype (Figure 4F; Table 1; Table S1). In fact, an increased insulin sensitivity could also contribute to the lowering of insulin secretion in GG carriers, since the insulin sensitivity index (ISI) increased significantly ($p = 0.02$). In CC carriers, ISI remained unchanged (Table 1). However, there was tendency for reduction in DI, but this did not reach statistical significance. Thus, the *MTNR1B* variant is unusual when it comes to its effect on insulin secretion and action, which both are lower in GG than in CC carriers (Jonsson et al., 2013). In contrast, the metabolic consequences of melatonin treatment in CC carriers were modest, restricted to fasting plasma glucose levels and CIR (Figure 4; Table 1). Thus, the quantitative effect of melatonin on insulin release was inhibitory and genotype specific.

The acute effects of melatonin on glucose tolerance in young healthy female athletes were recently reported (Garaulet et al., 2015). The subjects were divided into two groups with respect to the rs10830963 GC and CC genotypes in *MTNR1B*. Despite the low number of only heterozygous carriers of the risk G-allele (we examined homozygous GG-carriers), melatonin had a significant negative effect on glucose clearance during a 2 hr OGTT in the morning, but not in the night. There were no effects on basal melatonin levels or insulin levels during the challenge. Thus, both studies confirm that melatonin exerts effects on glucose metabolism in a genotype-specific fashion, which is evident in the morning, acutely and chronically.

Sleep Duration and Quality in Carriers of the *MTNR1B* Risk Variant

Melatonin is widely available over the counter and/or as a prescription drug for use as a sleep agent and in prevention of jet lag. We therefore examined whether the rs10830963 genotype affected sleep duration and quality. Although GG carriers reported slightly poorer sleep quality before melatonin treatment (Table 1), there was no difference between the GG and CC genotype carriers (Table 1) with respect to reported or recorded sleep quality in response to melatonin. In fact, sleep quality improved with melatonin treatment irrespective of rs10830963 genotype (Table S1), and, thus, altered sleep cannot account

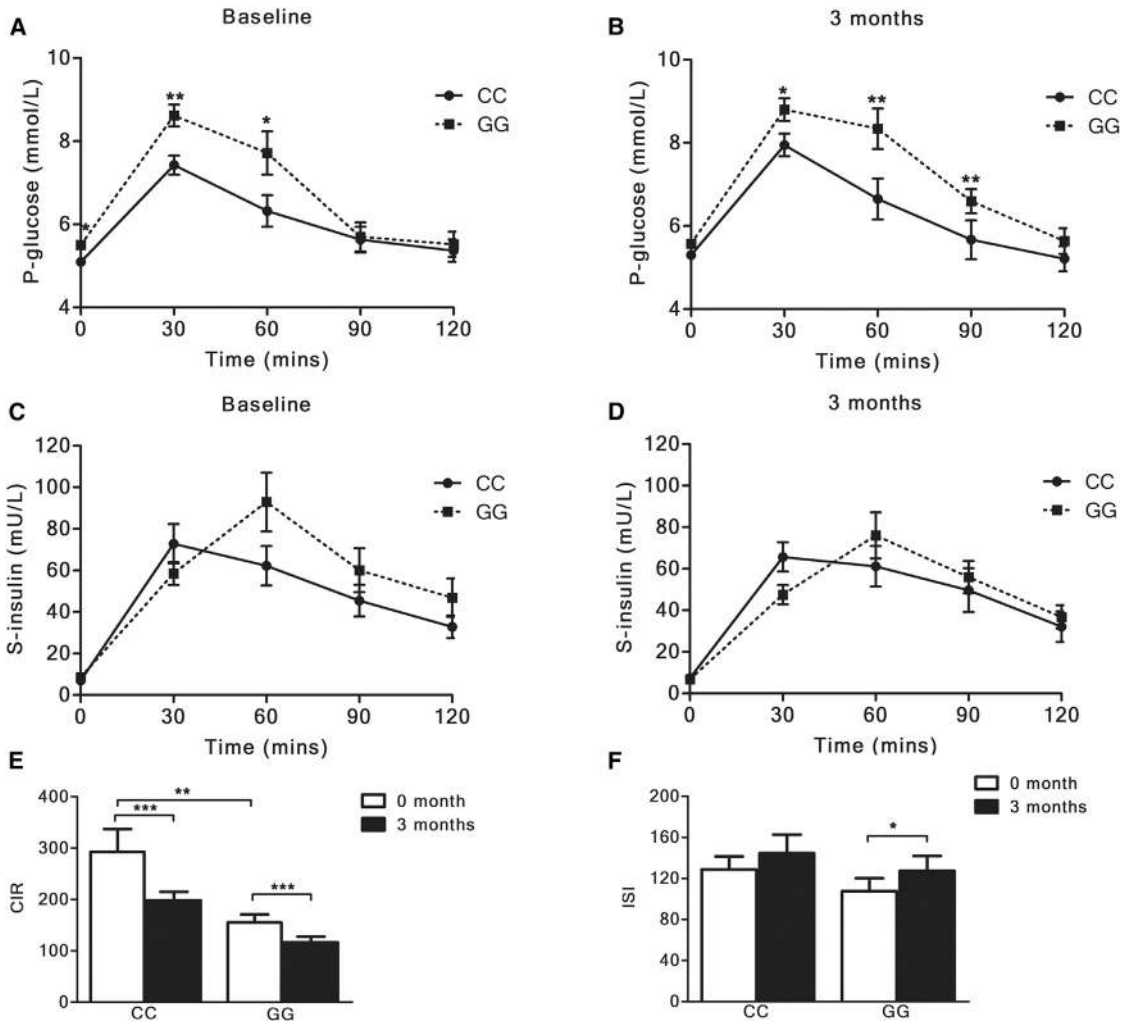

Figure 4. Melatonin Treatment Impairs Glucose Metabolism

Melatonin treatment impairs glucose metabolism more in carriers of the *MTNR1B* rs10830963 risk variant (GG; n = 23) than in nonrisk carriers (CC; n = 22) during an oral glucose tolerance test (OGTT; PPP-Botnia melatonin-study; MELAG).

(A) Mean plasma glucose levels during OGTT at baseline (CC, solid line; GG broken line); statistical comparisons between genotypes at each time point were made by Mann-Whitney U test; *p < 0.05, **p < 0.01; mean ± SEM.

(B) Plasma glucose levels during OGTT at 3 months (CC, solid line; GG broken line; statistics as in A).

(C) Plasma insulin response during OGTT at baseline (CC, solid line; GG broken line; mean ± SEM).

(D) Plasma insulin response during OGTT at 3 months (CC, solid line; GG broken line; mean ± SEM).

(E) Corrected early insulin response to glucose (CIR; mU*L/mmol^2). Statistical comparisons were made by Mann-Whitney U-test p < 0.01 = **, < 0.001 = ***; Mean ± SEM.

(F) Insulin sensitivity index (ISI; L^4/mmol^2*mU^2); statistical comparisons were made by Mann-Whitney U test; *p < 0.05; mean ± SEM.

See also Figure S4.

for the exaggerated effect of melatonin on insulin secretion in GG-carriers.

Given that the risk allele is very common in the population (about 30%) (Lyssenko et al., 2009), a widespread use of melatonin as sleep agent and in prevention of jet lag could potentially be more harmful than previously thought, particularly in carriers of the *MTNR1B* risk variant. In addition, there is concern that risk allele carriers may also be less suitable for nocturnal shift work, given that melatonin secretion is elevated in this group of workers, where metabolic disorders are more prevalent (Scheer et al., 2009). At this point our studies with respect to sleep do not support such concerns, although further study is clearly warranted. Also, genotype-specific impact of shift work would be very interesting to examine.

Concluding Remarks

The role of melatonin in control of whole-body metabolism and insulin release has been controversial (Mulder et al., 2009). While most previous studies have suggested an inhibitory effect of melatonin on insulin release (Peschke et al., 2006), some studies have also shown stimulatory effects (Kemp et al., 2002; Ramracheya et al., 2008). Moreover, both improved and impaired glucose

Table 1. Changes in Glucose Metabolism and Sleep Patterns after Melatonin Treatment in Homozygous Carriers of the *MTNR1B* GG or CC Genotypes

	GG Genotype			CC Genotype		
	Basal	3 Months	p Value	Basal	3 Months	p Value
CIR	142 (112)	124 (83)	0.008	238 (240)	194 (133)	0.008
HOMA-IR	1.6 (1.6)	1.4 (1.4)	0.71	1.3 (0.5)	1.4 (1.2)	0.71
ISI	98 (79)	120 (132)	0.02	133 (96)	129 (107)	0.18
DI	647 (852)	553 (663)	0.24	1,651 (952)	1,436 (646)	0.23
Glucagon 0 min	78 (23)	84 (38)	0.68	84 (25)	80 (28)	0.70
Glucagon 120 min	68 (31)	73 (21)	0.14	69 (17)	70 (22)	0.97
Ins/glucagon 0 min	0.09 (0.06)	0.07 (0.05)	0.0007	0.07 (0.05)	0.07 (0.07)	0.73
Ins/glucagon 120 min	0.46 (0.49)	0.36 (0.36)	0.02	0.35 (0.33)	0.28 (0.35)	0.71
Sleep duration (hr.min)	6.3 (1.0)	6.5 (0.7)	0.73	6.6 (1.1)	6.5 (1.3)	0.84
Sleep efficiency (%)	90.5 (11.7)	88.9 (6.9)	0.75	89.1 (4.8)	91.5 (4.6)	0.028
Wake-after-sleep-onset (min)	33.4 (39.6)	38.8 (27.0)	0.24	37.3 (24.4)	31.6 (19.0)	0.077
Pittsburgh Sleep Quality Index (sumscore range 0–21)	6.0 (5.0)	4.0 (4.0)	0.053	5.0 (2.0)	4.0 (2.0)	0.023
Epworth Sleepiness Scale (sumscore range 0–24)	5.0 (3.3)	5.0 (3.8)	0.28	6.0 (5.0)	5.0 (6.8)	0.073
Melatonin (pg/ml)	16.0 (30)	510.0 (726)	<0.0001	25.5 (31)	288.5 (550.0)	<0.0001

Study subjects were given 4 mg of melatonin at bedtime for 3 months. Results are data from patients carrying the GG-risk allele and the CC-nonrisk allele prior to and after the study, and expressed as medians (interquartile range). Ins, serum insulin (mU/l); gluc, plasma glucose (mmol/l); glucagon (ng/ml); HOMA-IR, homeostatic model assessment of insulin resistance; ISI, insulin sensitivity index ($L^4/mmol^2*mU^2$); CIR, corrected insulin response ($mU*L/mmol^2$); DI, disposition index. Sleep duration, sleep efficiency, and wake-after-sleep-onset are indices computed from data recorded by actigraphs; Pittsburgh Sleep Quality Index and Epworth Sleepiness Scale are self-reported with higher scores indicating poorer quality and higher chance of dozing. Nominal p values are shown. p = 0.486 and 0.078 for the difference between GG and CC genotypes carriers at baseline and 3 months, respectively. See also Table S1.

tolerance has been reported after melatonin therapy (Rubio-Sastre et al., 2014; Teodoro et al., 2014). The use of different species and experimental models may be a reason for these discrepancies. Another explanation could be that most human studies have not taken into account the genetic background of the study participants, and time of day for experiments has not been standardized. Here, using an array of experimental approaches in vivo and in vitro as well as clinical studies in humans, we arrived at the conclusion that the physiological role of melatonin in islets is to inhibit insulin release, most likely via a reduction in cAMP levels. A melatonin-mediated reduction in nocturnal insulin release, when melatonin levels are high but metabolic demands low, due to cessation of food intake, is perhaps a physiological and protective mechanism against nocturnal hypoglycemia.

Our group has previously reported that negative effects on both insulin secretion and sensitivity underlie the perturbations in glucose metabolism in carriers of rs10830963 G risk variant in *MTNR1B* (Jonsson et al., 2013). The human studies in this report translated the experimental findings into everyday life of the many people administering melatonin for sleep disorders or to prevent jet lag. While melatonin impaired insulin secretion in all carriers, this was more pronounced in homozygous carriers of the GG risk genotype. Moreover, the ratio of secretion of insulin to that of glucagon was reduced (Table 1), enabling opposite effects of these glucose-lowering (insulin) and glucose-raising (glucagon) hormones. The implications of these findings need to be further explored.

Worthy of note, our study is one of the first pharmacogenetic studies in the field of T2D, employing a recall-by-genotype design (Tang et al., 2014). It demonstrated that the effect of melatonin on glucose metabolism is dependent upon genotype. Although yet to be proven, carriers of the *MTNR1B* risk variant are likely to have lower cAMP levels in pancreatic β cells. In view of this, incretin-based therapy, which amplifies cAMP signaling in β cells, may be particularly well suited for these patients.

EXPERIMENTAL PROCEDURES

Expression of *MTNR1B* mRNA was examined by RNA sequencing, using Illumina HiSeq 2000, as previously described (Fadista et al., 2014). We analyzed *MTNR1B* expression in 204 batches of human islets from donors provided by the Nordic Center for Clinical Islet Transplantation in Uppsala, Sweden. The islets were cultured as previously described for 1–9 days prior to analysis (Fadista et al., 2014; Lyssenko et al., 2009).

Cell Culture and Adenovirus Transduction

Clonal insulin-secreting INS-1 832/13 β cells were cultured as previously described (Hohmeier et al., 2000). The cells were transduced with E1-deleted, replication-deficient adenovirus containing human *MTNR1B* cDNA under the control of the rat insulin promoter-2 (Ad-*Rip2-MTNR1B*) or an adenovirus containing green fluorescent protein (Ad-*Rip2-GFP*). For assay of insulin secretion, INS-1 832/13 β cells transduced with the respective adenoviruses were cultured for 48 hr followed by 1 hr static incubation with the indicated secretagogue. Insulin was measured by Coat-A-Count insulin radioimmunoassay (DPC, Los Angeles, CA).

Mt2 Knockout Mice

Melatonin receptor *Mtnr1b* knockout ($Mt2^{-/-}$) and WT mice were kindly provided by Professor David R. Weaver (University of Massachusetts Medical School, Worcester, MA, USA). The targeted deletion of the receptor to

generate *Mt2*$^{-/-}$ mice has been described previously (Jin et al., 2003). *Mt2*$^{-/-}$ mice carry a disrupted exon 1, resulting in expression of a nonfunctional receptor unable to bind melatonin.

Glucose and Insulin Tolerance Tests in Mice

Tolerance tests and clamps were performed in sedated mice. For IVGTT, mice were fasted for 10 hr followed by 1 g/kg glucose injections into a tail vein. For ITT, mice were fasted for 4 hr and given 0.75 mU/g intraperitoneal human insulin injections (Novo Nordisk, Clayton, NC). Hyperinsulinemic-euglycaemic clamps were performed as previously described (Kim et al., 2001).

Islet Isolation and Batch Incubation

Islets were as described previously (Fex et al., 2007) and handpicked under a stereomicroscope. Batches of four islets were incubated for 1 hr in KRBB buffer containing either 1 or 15 mM glucose. Aliquots of incubation medium were collected for measurement of insulin with ELISA (Insulin mouse ELISA; Mercodia, Uppsala, Sweden). Insulin from islets was extracted in acid ethanol at 4°C.

β Cell Mass

Pancreata were dissected in a standardized fashion, weighed, frozen on dry ice, and consecutive 10 μm thick sections cut. Insulin-immunostained area and total section area were measured in all islets in nine sections from three parts of the pancreas. β cell mass was calculated by multiplying area of insulin-positive cells/total pancreatic area with pancreatic weight. Islet number and mean islet size were also calculated on insulin-immunostained sections.

Analyses of cAMP

cAMP was extracted from frozen islets in an ethanol/dry ice bath and determined by cAMP enzyme-linked immunosorbent assay kit. cAMP was also measured in single living cells and intact pancreatic islets, using evanescent wave microscopy and a fluorescent translocation reporter as previously described (Dyachok et al., 2006; Tian et al., 2011).

Melatonin Intervention Trial

Participants from the population-based PPP-Botnia Study (Isomaa et al., 2010; Lyssenko et al., 2009), who had two copies of the rs10830963 risk allele (GG, n = 240), were matched with carriers of the WT allele (CC, n = 1059) for gender, age ±3 years, BMI ±1–2 kg/m^2, and glucose tolerance. Exclusion criteria were diabetes, pregnancy, poorly treated hypertension, glaucoma, coronary heart disease, arrhythmias, ulcer, panic attacks, psychosis, use of sleeping pills, beta or alpha blockers, antidepressive or antipsychotic medication, and abnormal creatinine or liver enzymes. A total of 23 GG and 22 CC carriers were recruited for the study, where they received 4 mg of melatonin (Circadin, Oy Leiras Finland Ab) once daily in the evening for 3 months. Before and after 3 months they underwent a 75 g OGTT with measurements of plasma glucose and serum insulin at 0, 30, 60, and 120 min, and plasma glucagon and serum melatonin at 0 min. Insulin sensitivity was evaluated by HOMA, and the ISI was calculated from the OGTT data. β cell function was assessed as the corrected insulin response (CIR) during the OGTT (Hanson et al., 2000), or a disposition index (DI = ISI × CIR) (Bergman et al., 2002; Matsuda and DeFronzo, 1999).

Sleep Quantity and Quality

Sleep was measured with actigraphs worn on the wrist for an average of 8 nights before the melatonin treatment and on average 7 nights before the second OGTT (Table S1). All participants provided completed sleep logs, including written sleep logs and electronic event markers of the bedtimes and waking times. Data were scored with Actiwatch Activity & Sleep Analysis version 7.38 software.

Statistical Analysis

Values were expressed as mean ± SEM or median and interquartile range. Statistical differences between means were assessed by Mann-Whitney U test, Wilcoxon matched-pairs signed rank test, or two-way ANOVA with Bonferroni's multiple comparison tests as indicated in the figure legends. The chi-square test was used to compare percentages of cells. In human data, Mann-Whitney U test and Wilcoxon matched-pairs signed rank tests were used. Nominal p values without correction for multiple testing are shown.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2016.04.009.

AUTHOR CONTRIBUTIONS

H.M. and L.G. conceived the study and wrote the manuscript with T.T., who was responsible for the clinical study. C.N., P. Singh, H.B., L.S., I.A., and Q.Y. performed the experimental studies. B.I., B.Ö., J.S., A.K.P., S.M., K.R., T.F., and L.H. participated in the human study. J.F., O.A., and P. Storm analyzed the RNA sequencing data. P.A. performed statistical analyses in the human studies. M.F., A.T., and N.W. directed experimental studies and cowrote the manuscript.

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