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Increased Melatonin Signaling Is a Risk Factor for Type 2 Diabetes

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Abstract:	Type 2 Diabetes (T2D) is a global pandemic. Genome-wide association studies (GWAS) 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
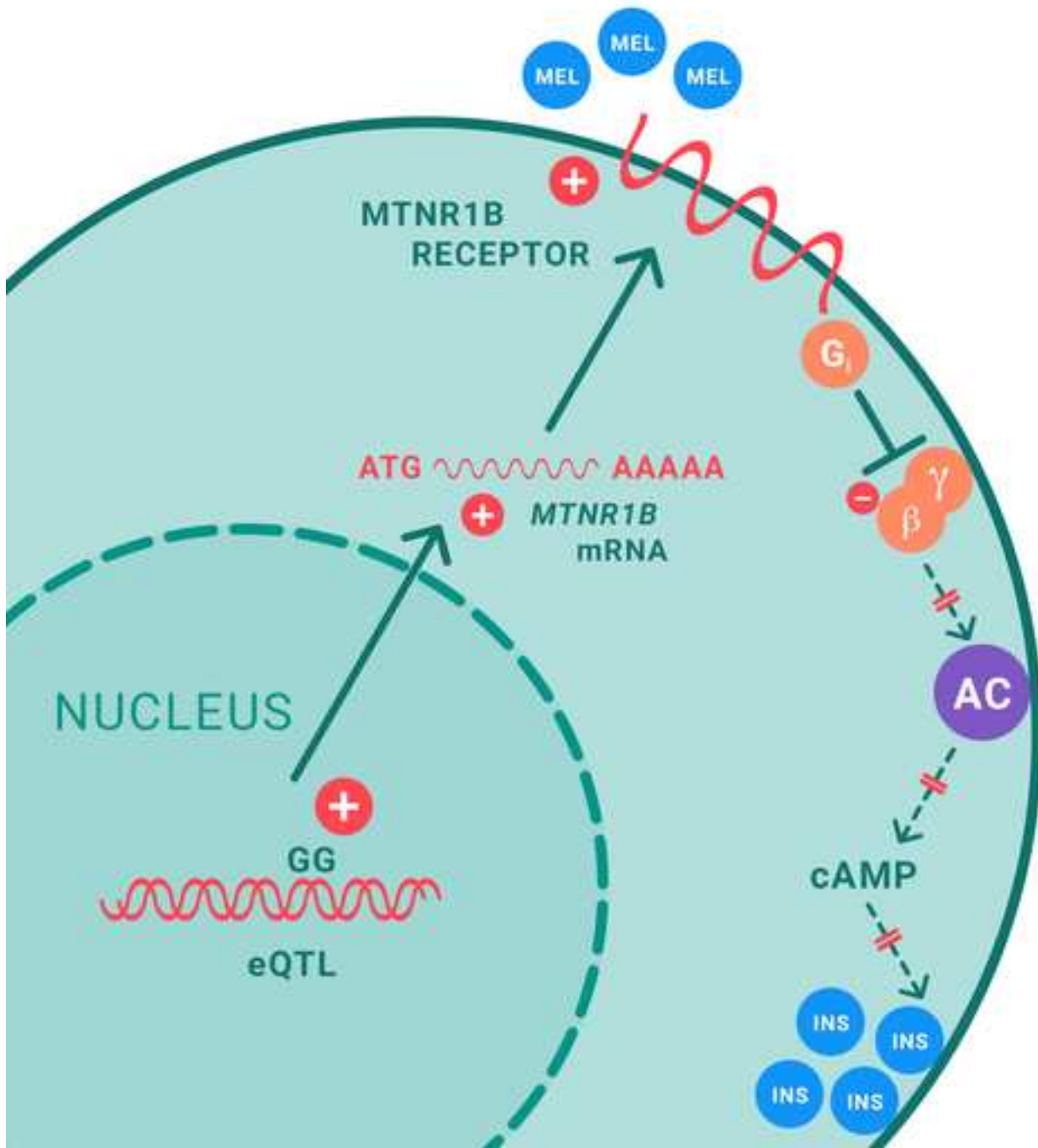
Increased melatonin signaling is a risk factor for Type 2 Diabetes

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SUMMARY

Type 2 Diabetes (T2D) is a global pandemic. Genome-wide association studies (GWAS) 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 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 (GWAS) 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 single nucleotide polymorphism (SNP) rs10830963 in the *MTNR1B* gene showed a stronger association with glucose levels in non-diabetic 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* knock out 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 (Fig. 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 establishe *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 (Muhlbauer 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* was 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 (Supplemental Fig. 1a). Insulin release in response to 16.7 mM glucose during a 1 h incubation of these cells was unaffected by *MTNR1B* overexpression (Fig. 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 (Fig. 1c). Moreover, *MTNR1B* overexpression slightly, but not significantly, blunted the initial glucose response (Fig. 1d) but significantly increased the fraction of melatonin-responsive cells and effect of the hormone (Fig. 1e-f). 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 (Fig. 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 knock out 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 (Supplemental Fig. 2a-b). An intravenous glucose tolerance test (IVGTT; Fig. 2a-b; Supplemental Fig. 2c-e) revealed increased insulin secretion during the challenge. This exaggerated response to glucose was more robust in female than in male mice (Supplemental Fig. 2f-h). Despite elevated insulin levels, plasma glucose levels and glucose elimination rates (Kg) in *Mt2$^{-/-}$* mice remained unchanged (Fig. 2c).

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 (Fig. 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 (Fig. 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 (Fig. 2e; Supplemental Fig. 3a). 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 (Fig. 2f), these findings suggest impaired insulin sensitivity at the level of the liver. Glucose uptake in muscle trended towards a decrease while that in fat remained unaltered (Fig. 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 (Supplemental Fig. 3b, c).

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 knock out mice have been reported.

β-cell mass, insulin secretion and cAMP in melatonin receptor 1B/MT2 knock out mice

To further clarify why insulin secretion *in vivo* is exaggerated in the *Mt2*^{-/-} mice, we examined β-cell mass. *Mt2*^{-/-} mice displayed greater β-cell mass compared to WT mice (Fig. 3a). The increased β-cell mass was due to an increase in number of islets but not in mean islet size (Fig. 3b, c). Accordingly, insulin content was similar in *Mt2*^{-/-} and WT islets (Fig. 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 (Fig. 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 (Fig. 3f). Moreover, on-line recordings with the fluorescent cAMP reporter revealed a more pronounced initial glucose-induced cAMP elevation in islet cells from *Mt2*^{-/-} mice (Fig. 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 (Fig. 3h, i). 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-

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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.

Knock out mice for melatonin receptors have previously been studied with mixed results. Isolated islets from $Mt1^{-/-}$ mice as well as double knock out mice for *Mt1* and *Mt 2* lose the inhibitory effect of melatonin on insulin secretion (Muhlbauer et al., 2012). In $Mt2^{-/-}$ mice, there is a 40% non-significant 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 h 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* (Muhlbauer 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, data represent a chronic situation where signaling from MTNR1B is exaggerated, and that incubations of cells have largely been for 6 h. 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 non-diabetic individuals with two copies of the risk allele (GG) and 22 carriers of two copies of the non-risk allele (CC) were administered 4 mg of melatonin at bedtime for three 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 vs. CC genotype carriers (Fig. 4a, c; 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 three months of melatonin treatment, there was a clear decrease in first-phase insulin release and an increase in glucose concentrations in all subjects (Supplemental Fig. 4; Supplemental Table 1). This was particularly clear in GG carriers (Fig. 4b, d; Table 1), despite identical dosage of melatonin and similar plasma melatonin concentrations in GG and CC carriers. When compared with base line data, measures of insulin secretion were lower in the GG carriers after melatonin treatment (Fig. 4e,f; Table 1), particularly the corrected insulin response (CIR) during the first 30 min of the OGTT (P=0.008; Fig 4e). Notably, insulin sensitivity increased after melatonin treatment regardless of genotype (Fig. 4f; Table 1, Supplemental Table 1). 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 contrast, a trend to decrease was observed in CC carriers (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 (Fig. 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 h oral glucose tolerance test 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 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 (Supplemental Table 1), and, thus, altered sleep cannot account 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 wide-spread 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 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 every-day 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

Human islets and RNA sequencing

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 h followed by 1 h static incubation with the indicated secretagogue. Insulin was measured by Coat-A-Count insulin radioimmunoassay (DPC, Los Angeles, CA).

Mt2 knock out mice

Melatonin receptor *Mtnr1b* knockout ($Mt2^{-/-}$) and wild type (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 non-functional receptor unable to bind melatonin.

Glucose and insulin tolerance tests in mice

Tolerance tests and clamps were performed in sedated mice. For intravenous glucose tolerance tests (IVGTT), mice were fasted for 10-h followed by 1 g/kg glucose injections into a tail vein. For ITT mice were fasted for 4 h 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 4 islets were incubated for 1 h 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 9 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, abnormal creatinine or liver enzymes. 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 three months. Before and after 3 months they underwent a 75g OGTT with measurements of plasma glucose and serum insulin at 0, 30, 60, 120 min, and plasma glucagon and serum melatonin at 0 min. Insulin sensitivity was evaluated by HOMA and the insulin sensitivity index (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 x 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 (Supplemental Table 1). 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 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 for this article is available online.

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.Si, 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.St 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 co-wrote the manuscript.

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FIGURE AND TABLE LEGENDS

Fig. 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. See also Supplemental Fig. 1.

(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 Green Fluorescent Protein 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 4 experiments.

(d) On-line 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 larger response in *MTNR1B*-expressing cells is likely due to a higher steady-state cAMP level in these INS-1 832/13 β-cells.

Fig. 2. *Mt2⁻ᐟ⁻* mice exhibit increased insulin release and reduced hepatic insulin sensitivity. See also Supplemental Fig. 2-3.

(a) Intravenous glucose tolerance test (IVGTT) in 12 week old female WT (black; n = 12) and *Mt2⁻ᐟ⁻* (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⁻ᐟ⁻* (white; n = 4) mice during IVGTT in (b). 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⁻ᐟ⁻* (white; n = 4) female mice during IVGTT in (b). Glucose elimination rate (K) in %/min calculated from linear regression over the first 10 minutes after glucose injection. Mean ± SEM.

(d) Insulin tolerance test (ITT) in 24 week old WT (black; n = 5) and *Mt2⁻ᐟ⁻* (white; n = 15) female mice. Glucose values expressed as % glucose, where glucose at 0 minutes is 100%. Mean ± SEM.

(e) Hyperinsulinemic-euglycaemic clamp in 26 week old WT (black; n = 8) and *Mt2⁻ᐟ⁻* (white; n = 6) female mice. Ra = Rate of appearance of 3-³H- glucose during basal period = whole body glucose turnover = endogenous hepatic glucose production (HGP); Rd = Rate of disappearance of 3-³H- 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 (f). Mann-Whitney U-test: $p= 0.05$. Mean ± SEM.

Fig. 3. *Mt2⁻ᐟ⁻* mice exhibit increased insulin release due to increased β-cell mass and exaggerated cAMP responses.

(a) β-cell mass in 16-19 week old male and female WT (black; n = 6) and *Mt2⁻ᐟ⁻* (white; n =10) mice. β-cell mass is calculated as islets number/total area X pancreas weight. Mann-Whitney U-test: $p=0.05$; Mean ± SEM.

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(b) Mean islet size in 16-19 week old WT (black; n = 6) and *Mt2^-/-* (white; n =10) male and female mice. Mann-Whitney U-test: p<0.05=*; Mean ± SEM.

(c) Number of islets / mm^2 area calculated as islet area/total area in 16-19 week old WT (black; n = 6) and *Mt2^-/-* (white; n =10) male and female mice. Mean ± SEM.

(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 (1mM glucose) from islets of 12 week old WT (black; n = 7) and *Mt2^-/-* (white; n = 9) female mice after 1 h 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 5.5mM glucose. Mann-Whitney U-test: p< 0.01 = **. Mean ± SEM.

(g) On-line 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 2 (WT) and 3 (*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 (2 isolations) and 22 cells from *Mt2^-/-* mice (3 isolations). p<0.001=*** for difference (2-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 (2-tailed Mann-Whitney U-test).

Fig. 4. Melatonin treatment impairs glucose metabolism more in carriers of the *MTNR1B* rs10830963 risk variant (GG; n=23) than in non-risk carriers (CC; n=22) during an oral glucose tolerance test (OGTT; PPP-Botnia melatonin-study; MELAG). See also Supplemental Fig. 4.

(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=*, <0.01=**; Mean ± SEM.
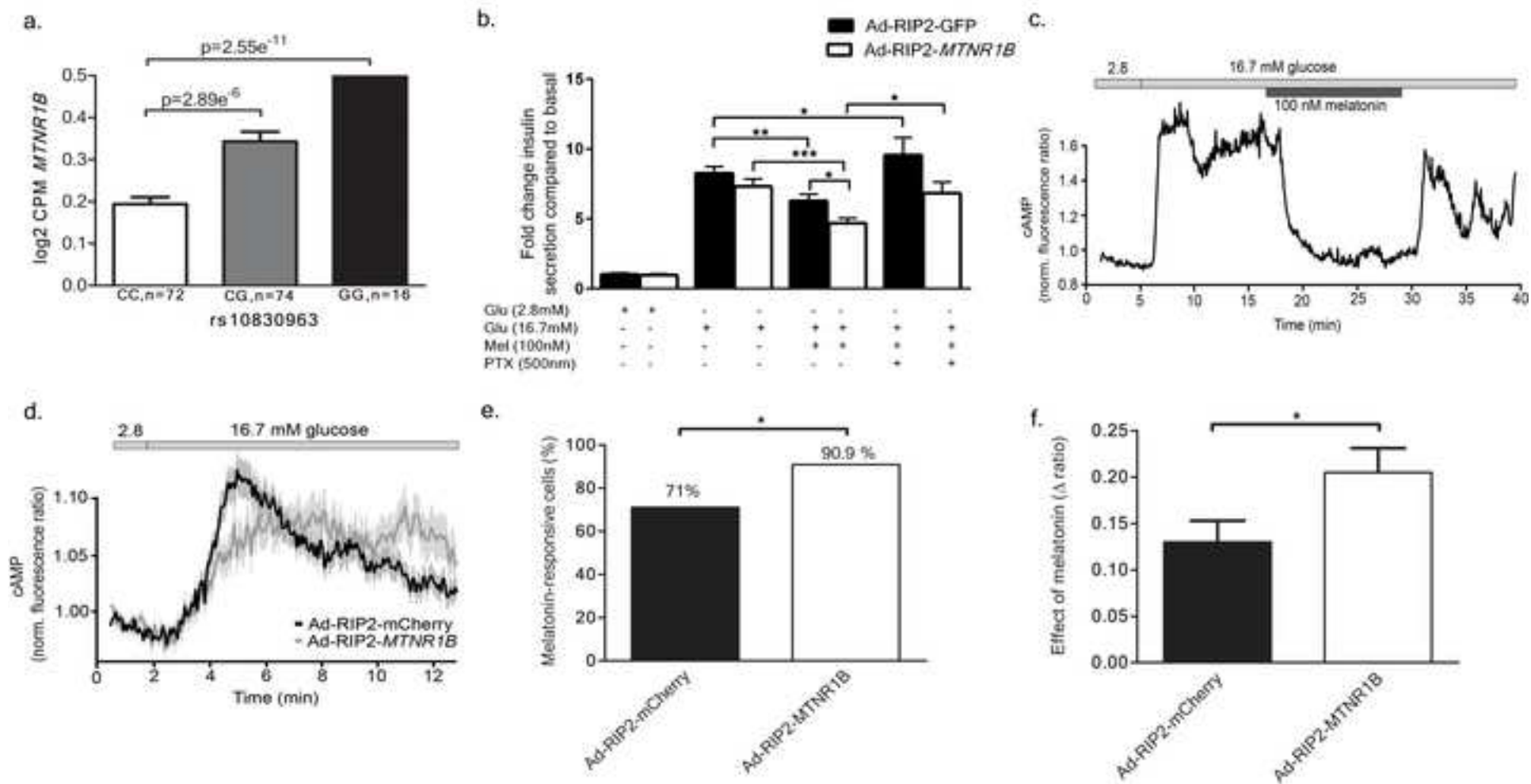
(b)　Plasma glucose levels during OGTT at three months (CC, solid line; GG broken line; statistics as in a)

(c)　Plasma insulin response during OGTT at baseline (CC, solid line; GG broken line; Mean ± SEM)

(d)　Plasma insulin response during OGTT at three 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.
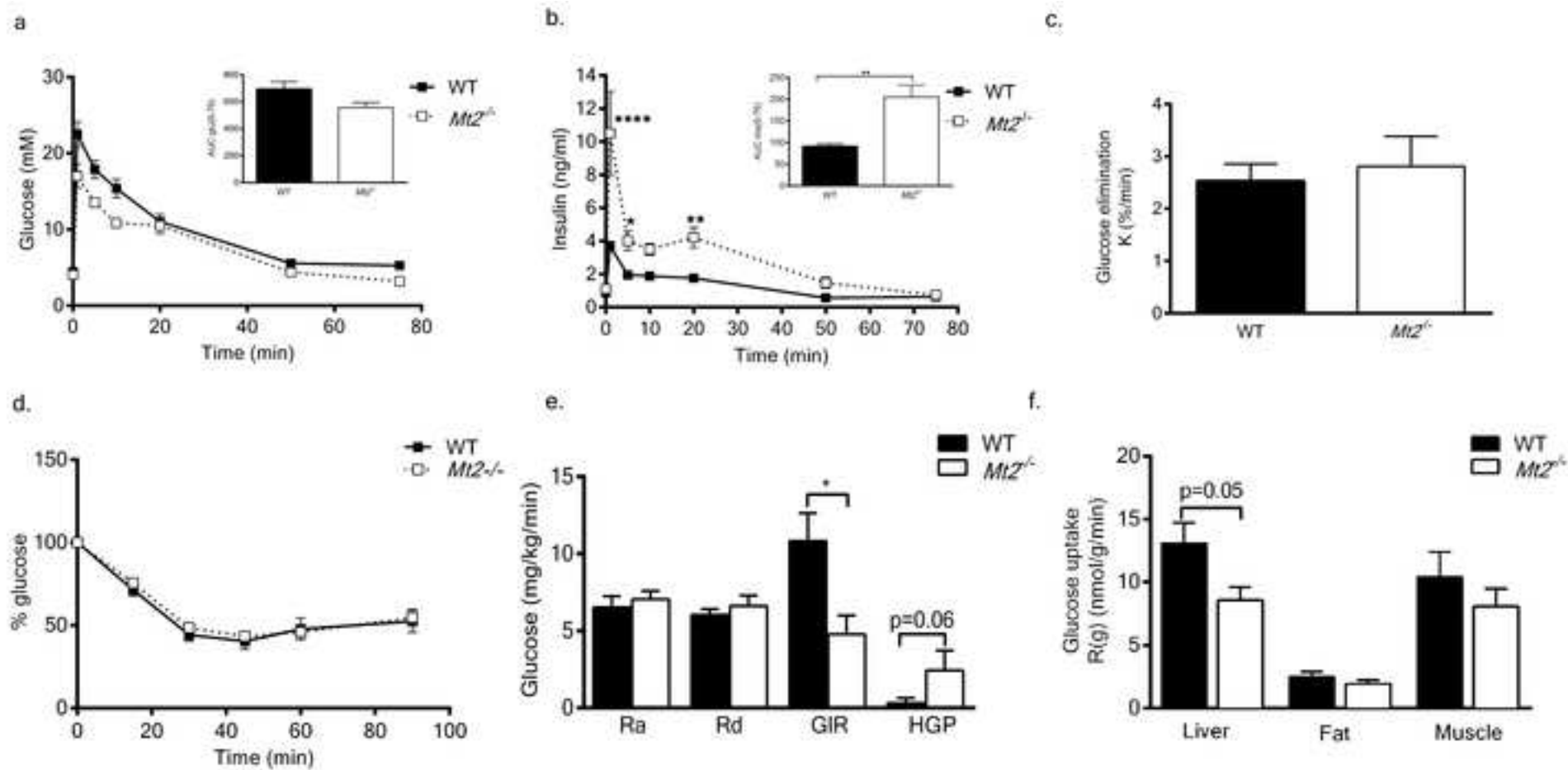
Table. 1 Study subjects were given 4 mg of melatonin at bedtime for three months. Results are data from patients carrying the GG-risk allele and the CC-non-risk allele prior to and after the study, and expressed as medians (interquartile range). Ins – serum insulin (mU/l); gluc – plasma glucose (mmol/l); 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 Supplemental Table 1.
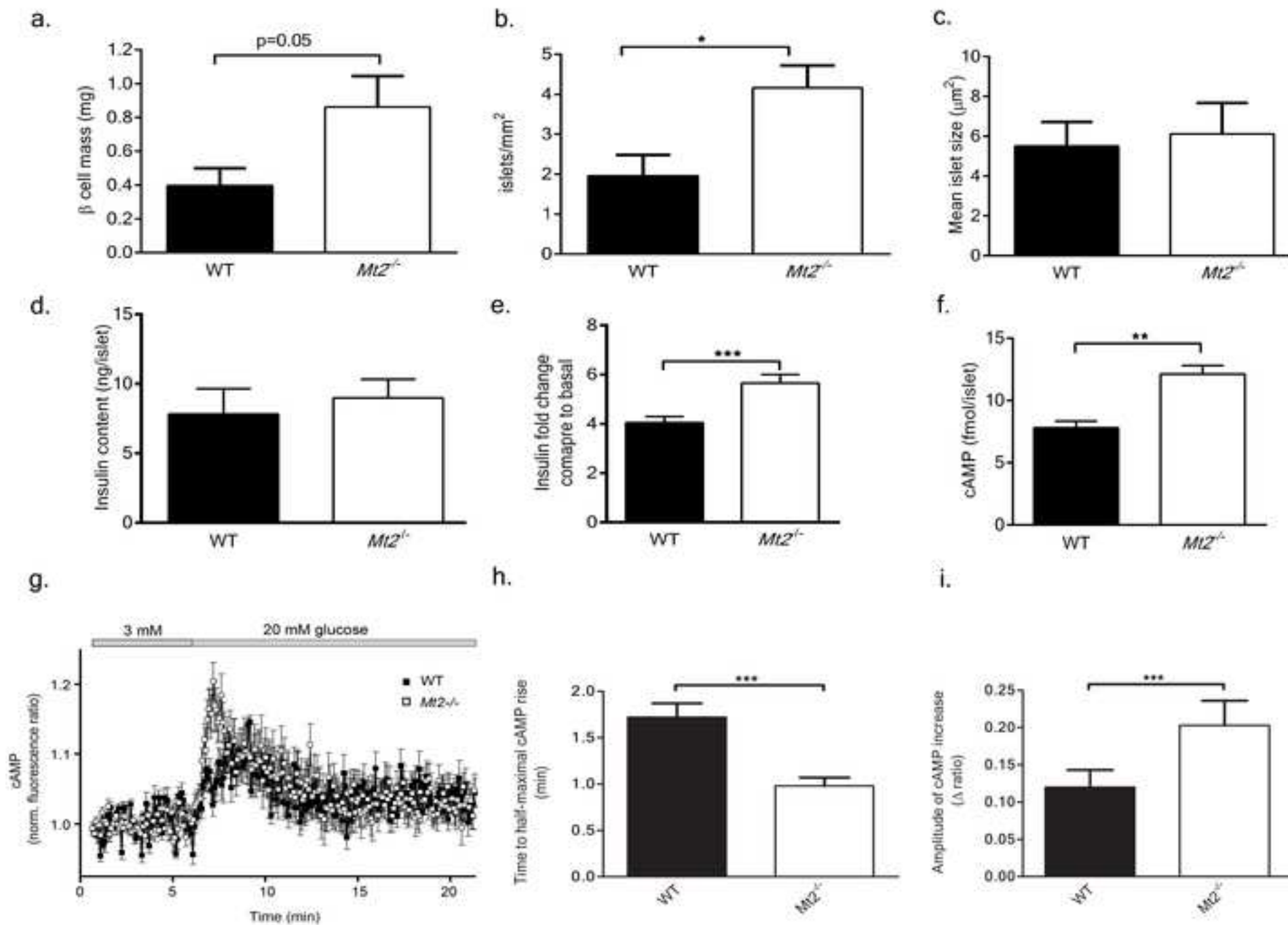
TABLES

Table 1. Changes in glucose metabolism and sleep patterns after melatonin treatment in homozygous carriers of the *MTNR1B* GG or CC genotypes
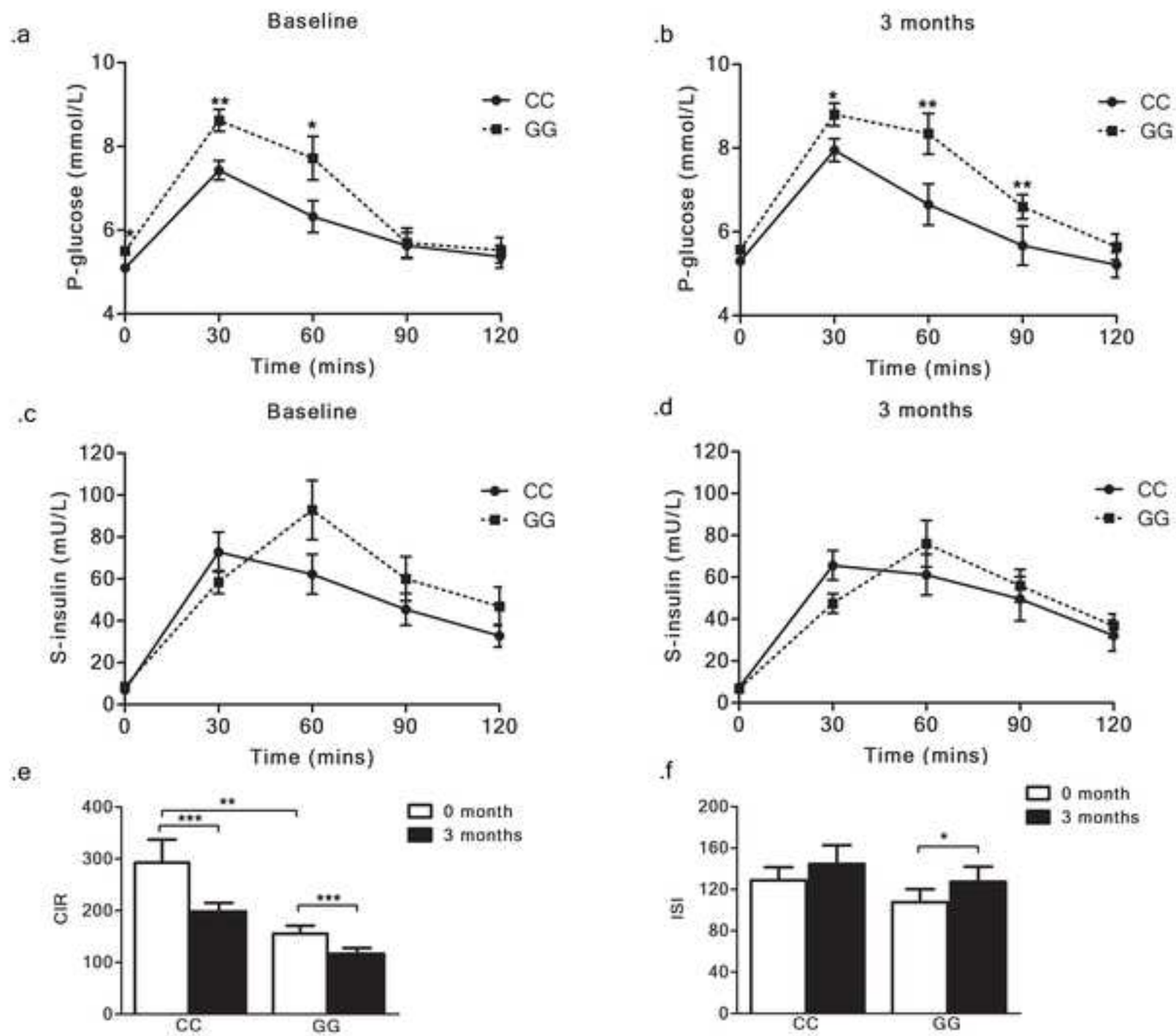
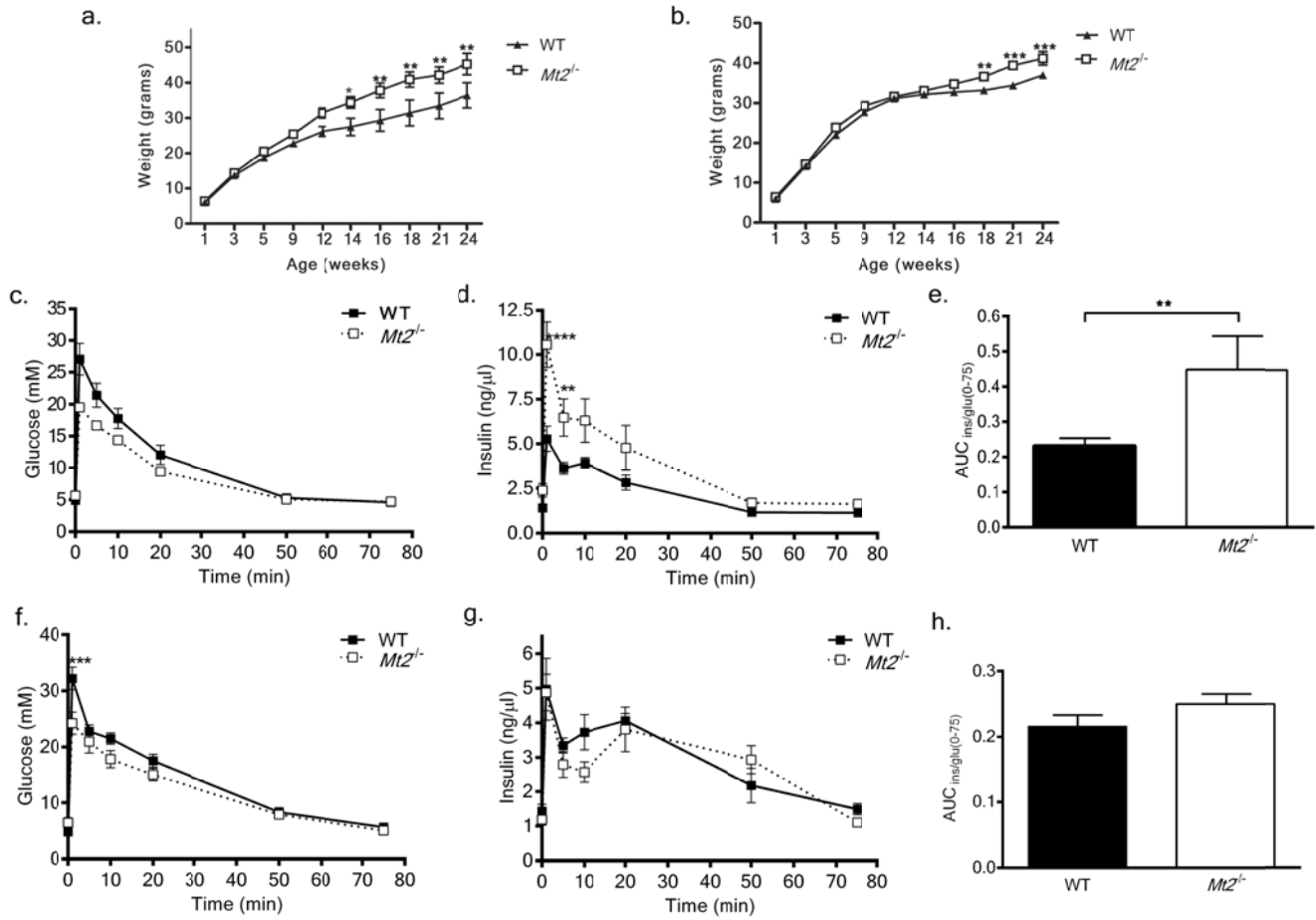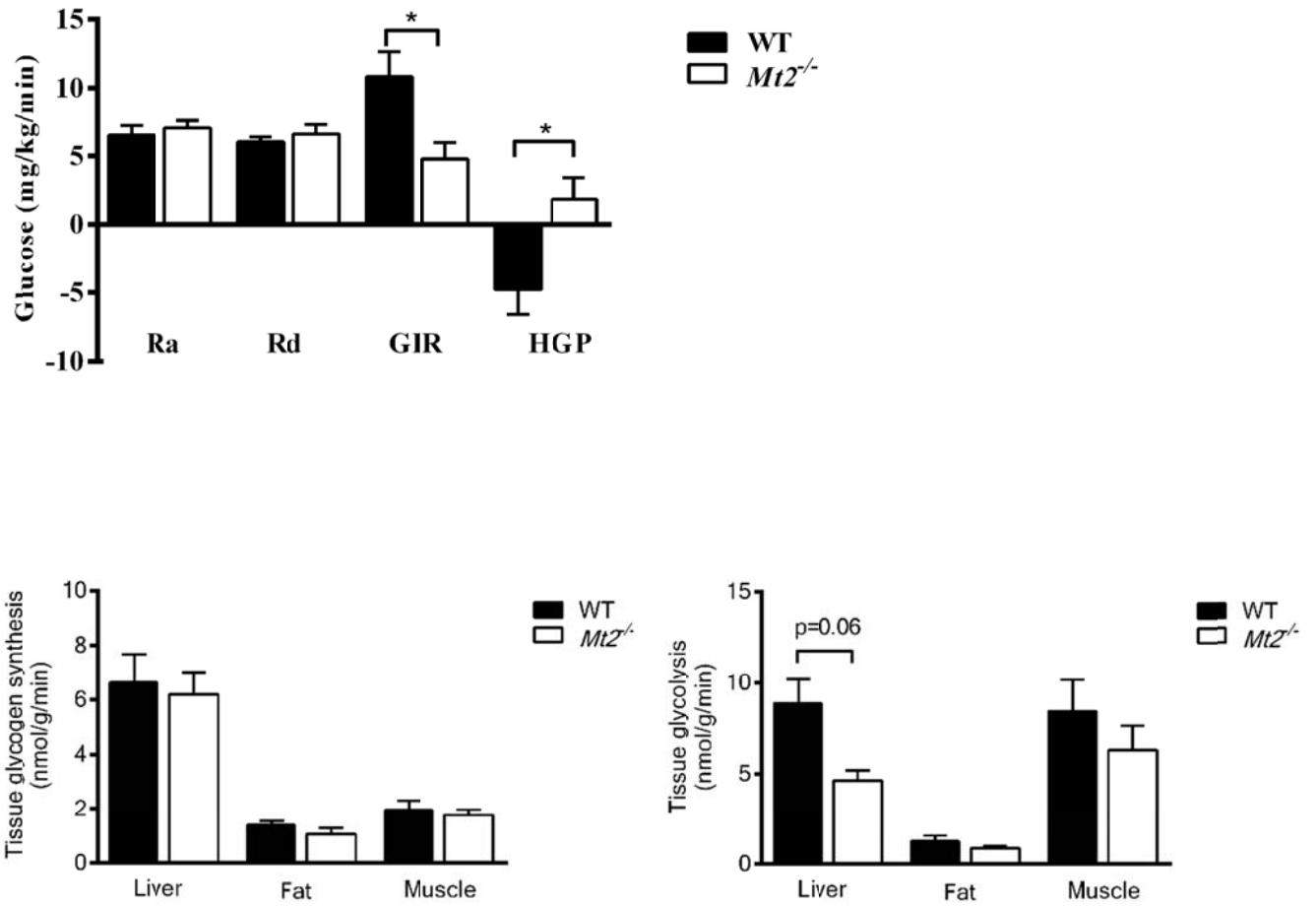|	GG genotype			CC genotype		
	Basal	3 months	P-value	Basal	3 months	P-value
CIR	142 (112)	124 (83)	0.008	238 (240)	194 (133)	0.008
HOMA-IR	1.6 (1.6)	1.4 (1.4)	0.71	1.3 (0.5)	1.4 (1.2)	0.71
ISI	98 (79)	120 (132)	0.02	133 (96)	129 (107)	0.18
DI	647 (852)	553 (663)	0.24	1651 (952)	1436 (646)	0.23
Glucagon 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 (hrs.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

Figure 2

Figure 3

Figure 4

Click here to download Figure Figure 4.tif
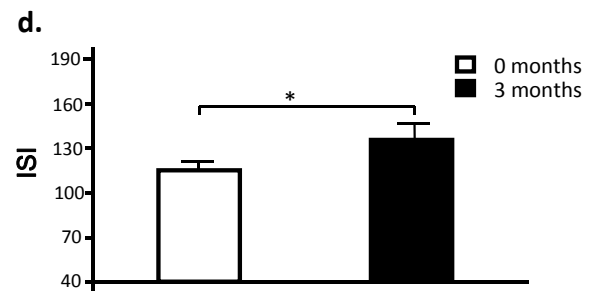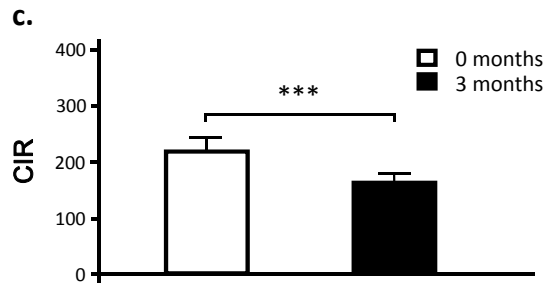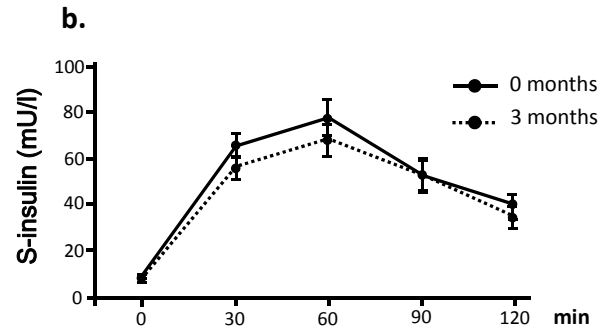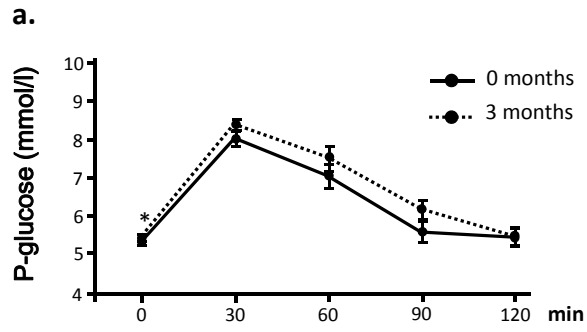
Supplemental Fig. 1

a.

b.
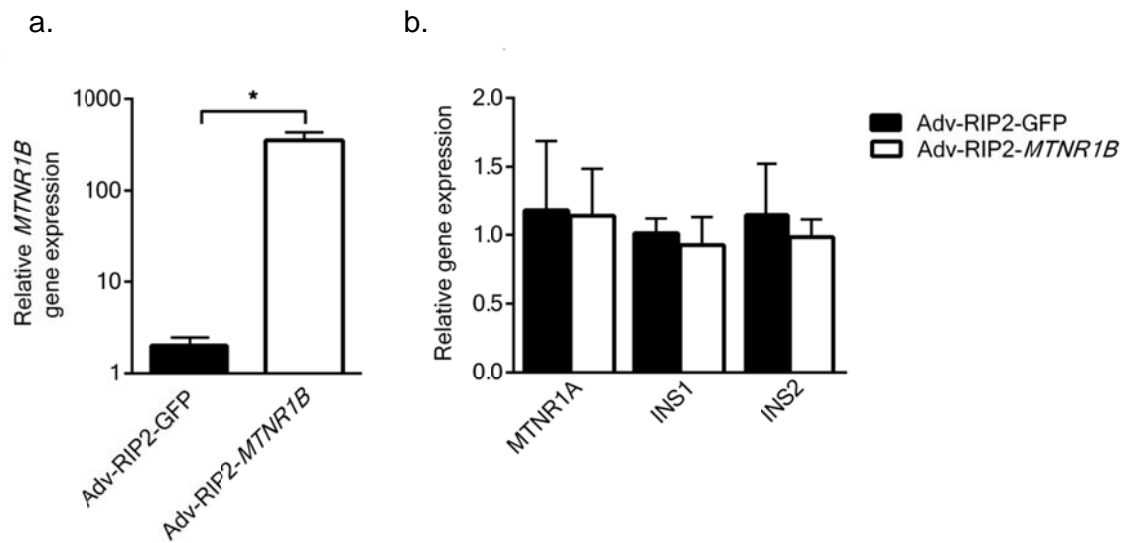
Supplemental Fig. 2

Supplemental Fig. 3

Supplemental Fig. 4

Supplemental Figure Legends

Fig. S1, related to Fig. 1. *MTNR1B* overexpression in INS-1 832/13 β-cells.

(a) Gene expression of *MTNR1B* in adv-RIP2-*MTNR1B* compared to adv-RIP2-GFP transduced β-cells. Expression is shown as relative expression. Mann-Whitney U-test: $p<0.05$=*; Mean ± SEM.

(b) Gene expression of *MTNR1A, INS-1 and INS-2* in adv-RIP2-*MTNR1B* infected β-cells compared to control virus-transduced cells. Expression is shown as relative expression for each individual transcript and can be compared between conditions. Mean ± SEM.

Fig. S2, related to Fig. 2. Glucose metabolism in male and female WT and *Mt2*^{-/-} mice.

(a) Weight development in WT (black; n = 5) and *Mt2*^{-/-} (white; n = 3) female mice. Two-way ANOVA with Bonferroni's multiple comparisons *post hoc* was used for testing statistical significance between genotypes at different time points. $p<0.01$=*, $p<0.01$=**; Mean ± SEM.

(b) Weight development in WT (black; n = 4) and *Mt2*^{-/-} (white; n = 3) male mice. Statistics as in (a); $p<0.01$=**, $p<0.001$=***; Mean ± SEM.

(c) Intravenous glucose tolerance test (IVGTT; glucose levels) at 24 weeks in WT (black; n = 9) and *Mt2*^{-/-} (white; n = 9) female mice.

(d) Plasma insulin levels at 24 weeks during IVGTT in (b). Statistics as in (a). $p<0.01$=**, < 0.0001=****; Mean ± SEM.

(e) Ratio area under curve (AUC) insulin/AUC glucose during IVGTT in (b). Mann-Whitney U-test; $p< 0.01$ =**; Mean ± SEM.

(f) Intravenous glucose tolerance test (glucose levels) at 24 weeks in WT (black; n = 6) and *Mt2*^{-/-} (white; n = 10) male mice. Statistics as in (a). $p<0.01$=***; Mean ± SEM.

(g) Plasma insulin levels at 24 weeks during IVGTT (glucose levels) in (e). Mean ± SEM.

(h) Ratio AUC insulin/AUC glucose during IVGTT in (e). Mean ± SEM.

Fig. S3, related to Fig. 2. Hyperinsulinemic-euglycaemic clamp.

(a) Hyperinsulinemic-euglycaemic clamp in 26 week 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. For HGP, also negative values were included in contrast to Fig. 1g, where negative values of HGP were set to zero. A typical reason for negative values is that steady state may not have been reached. In that case, the most conservative way to present data is to set these values to zero, since HGP physiologically cannot be less than zero. Mann-Whitney U-test: $p<0.05$=*. Mean ± SEM.

(b) Insulin-stimulated tissue glycogen synthesis during clamp for liver, fat and muscle in 26 week old WT (black; n = 8) and $Mt2^{-/-}$ (white; n = 6) female mice. Mean ± SEM.

(c) Insulin-stimulated tissue glycolysis for liver, fat and muscle calculated as glucose uptake-glycogen synthesis in 26 week old WT (black; n = 8) and $Mt2^{-/-}$ (white; n = 6) female mice during clamp. Mann-Whitney U-test: p= 0.06; Mean ± SEM.

Fig. S4, related to Fig. 4. Three months of melatonin treatment impairs glucose metabolism in non-diabetic subjects during oral glucose tolerance test regardless of genotype. (n=45 (GG + CC); OGTT; PPP-Botnia melatonin-study; MELAG).

(a) Plasma glucose levels during OGTT at baseline (solid line) or at three months (broken line; Mean ± SEM)

(b) Plasma insulin response during OGTT at baseline (solid line) or at three months (broken line; Mean ± SEM)

(c) Corrected early (30 min) insulin response to glucose (CIR; mU*L/mmol^2). Mann-Whitney U-test; $p< 0.001$= ***, Mean ± SEM.

(d) Insulin sensitivity index (ISI; ln transformation; L^4/mmol^2*mU^2). Mann-Whitney U-test; $p<0.05$= *, Mean ± SEM.

Supplemental Table 1, related to Table 1: Effect of melatonin on glucose metabolism and sleep patterns in all subjects

	Basal	3 months	P-value
CIR	183 (162)	142 (106)	0.0002
HOMA-IR	1.6 (1.2)	1.4 (1.2)	0.08
ISI	113 (89)	127 (119)	0.014
DI	1211 (1021)	995 (1041)	0.12
Glucagon 0 min	79 (23)	83 (30)	0.98
Glucagon 120 min	68 (19)	71 (20)	0.26
Ins/glucagon 0 min	0.08 (0.05)	0.07 (0.06)	0.03
Ins/glucagon 120 min	0.38 (0.43)	0.34 (0.34)	0.05
Sleep duration (hrs.min)	6.6 (1.0)	6.5 (0.8)	0.68
Sleep efficiency (%)	90.1 (7.4)	89.7 (5.0)	0.14
Wake-after-sleep-onset (min)	36.8 (26.0)	32.0 (21.0)	0.048
Pittsburgh Sleep Quality Index (sumscore range 0-21)	5.0 (3.0)	4.0 (3.0)	0.004
Epworth Sleepiness Scale (sumscore range 0-24)	5.0 (5.0)	5.0 (5.0)	0.036
Melatonin (pg/ml)	19 (31)	290 (704)	<0.0001

Study subjects were given 4 mg of melatonin at bedtime for three months. Results are data from all patients irrespective of genotype prior to and after the study, and expressed as medians (interquartile range). Ins – insulin (mU/l); gluc – glucose (mmol/l), glucagon ng/ml; CIR – corrected insulin response (mU*L/mmol^2); HOMA-IR – homeostatic model assessment for insulin resistance; ISI – insulin sensitivity index (L^4/mmol^2*mU^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.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Human islets and RNA sequencing

Human islets from 204 islet donors were from the Nordic Center for Clinical Islet Transplantation (courtesy of Professor Olle Korsgren, Uppsala University, Sweden), and processed at the Human Tissue Laboratory, Lund University Diabetes Centre. Purity varied from 23% to 90%, as determined by supravital dithizone staining. The islets were cultured as previously described for 1-9 days prior to analysis (Fadista et al., 2014; Lyssenko et al., 2009). All islet donors had consented to donate organs for medical research. The procedures were approved by the human ethical committees in Uppsala and Lund.

RNA sequencing libraries were generated and sequenced on Illumina HiSeq 2000, using paired-end chemistry and 100-bp cycles to an average depth of 32M read pairs/sample as previously described (Fadista et al., 2014). Data were $\log^2$-transformed and normalized, using trimmed mean of M-values as implemented in the R-package edgeR.

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 recombinant adenovirus (Vector BioLabs, Philadelphia, PA, USA). The adenovirus was E1-deleted, replication-deficient and contained human *MTNR1B* cDNA under the control of a rat insulin promoter-2 (Ad-*Rip2-MTNR1B*). The control virus consisted of a similar adenovirus containing green fluorescent protein (Ad-*Rip2-GFP*). Adenoviral infection was performed on 2×10^5 cells, which had been cultured for 24 h prior to infection. Cells were incubated with adenoviral aliquots at multiplicity of infection (MOI) of 10 for 4 h before addition of fresh culture medium. The overexpression of *MTNR1B* was confirmed by calculating its mRNA levels by real-time PCR after reverse transcription as described (Malmgren et al., 2009). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA levels were used for normalization between samples.

INS-1 832/13 β-cells transduced with the respective adenoviruses were cultured for 48 h before experiments. For insulin secretion, the cells were washed and pre-incubated in secretion assay buffer (SAB) containing (mM) 114 NaCl, 4.7 KCl, 1.2 KH$_2$PO4, 1.16 MgSO$_4$, 20 HEPES, 25.5 NaHCO$_3$, 2.5 CaCl$_2$ at pH 7.2 with 2 g/l BSA and 2.8 mM glucose for 2 h at 37°C. The buffer was replaced with fresh SAB containing indicated concentrations of glucose, melatonin and pertussis toxin (PTX). After 1 h of incubation, the media were collected and insulin levels measured by RIA, using Coat-A-Count insulin radioimmunoassay (DPC, Los

Angeles, CA). The insulin secretion was normalized to the cellular protein content, determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Mouse studies

Age-matched male and female mice of melatonin receptor *Mtnr1b* knockout (*Mt2*^{-/-}) and wild type (WT) mice were used. These 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). These mice carry a disrupted exon 1, resulting in expression of a non-functional receptor unable to bind melatonin. The genotype of the knockout lines was verified by analysis of genomic DNA using PCR. Mice were housed at room temperature regulated at $22 \pm 2°C$, with a 12-h light–dark cycle. Standard chow pellets and water were given freely, except during the period of food deprivation. All experimental protocols for animals were approved by the local animal ethical committee of Lund/Malmö and complied with the guidelines of the Federation of European Laboratory Animal Science Association (FELASA).

Glucose and insulin tolerance tests in mice

All tolerance tests were performed following sedation of mice, using 0.25 mg midazolam (Midazolam Panpharma ®; Fougères, France) in combination with 0.5 mg fluanison and 0.02 mg fentanyl (Hypnorm ®; VetPharma Ltd, Leeds, UK). For intravenous glucose tolerance tests (IVGTT), mice were fasted for 10-h followed by 1 g/kg glucose injections into a tail vein. For ITT mice were fasted for 4 h and given 0.75 mU/g intraperitoneal human insulin injections (Novo Nordisk, Clayton, NC). The mice were bled retro-orbitally to collect blood at indicated time points. Plasma glucose concentrations were measured by the Infinity Glucose Oxidase Liquid Stable Reagent (Thermo Electron Corporation, Victoria, Australia). Insulin was assayed by Ultrasensitive enzyme-linked immunosorbent assay (ELISA; Mercodia, Uppsala, Sweden).

Hyperinsulinemic-euglycaemic clamp in mice

Hyperinsulinemic-euglycaemic clamps were performed, using chronically cannulated, fasted (4 h), mice as previously described (Kim et al., 2001). The protocol included a 100 min tracer equilibrium period (−100 to 0 min) and a 90 min experimental period (0 to 90 min). At t = -100 min, infusion of 3-^{3}H-glucose was started at 0.05 µCi/min (Glucose D-(3-3H); Perkin Elmer, Sweden). At t = 0 min, an insulin bolus of 150 mU/kg was given and then continued at a constant rate at 3.5 mU/kg/min throughout the clamp (Actrapid 100 IU/IE/ml; Novo Nordisk, Denmark). Simultaneously, the infusion rate of 3-^{3}H glucose was increased to 0.1 µCi/ min, which was maintained until the end of experiment to avoid changes from equilibration period.

Euglycaemia ($\approx$ 6.3 mM) was maintained during clamps by measuring blood glucose every 10 min starting at t= 0 min and infusing 20% glucose as necessary. To estimate insulin-stimulated glucose transport activity and metabolism in skeletal muscle, liver and fat, 2-deoxy-D-[^{14}C]glucose (Deoxy-D-glucose, 2-(1-^{14}C)-, Perkin Elmer, Sweden) was administered as a bolus (10 μCi) at 35 min before the end of clamp. Blood samples were taken at 60, 65, 70, 75, 80, and 90 min after the start of the clamp for the determination of plasma 3-^{3}H glucose, 2-[^{14}C] DG, and ^{3}H$_2$O concentrations. Plasma glucose during the clamp was analyzed using a glucose meter (ACCU-CHEK; Aviva, Sweden). Insulin was measured by mouse ELISA kit. Analysis of 3-^{3}H glucose measurements and calculation of glucose-disposal rates and hepatic glucose production rates were performed as described earlier (Kim et al., 2001). At the end of the clamp, mice were killed by CO_2 inhalation.

Islet isolation and batch incubation

Islets were isolated using collagenase perfusion method described previously (Fex et al., 2007), and handpicked under a stereomicroscope (Stereozoom GZ7, Leica, Buffalo, New York, USA). After isolation, islets were cultured overnight in RPMI-1640 medium supplemented with 11 mM glucose and 10% (vol/vol) fetal bovine serum. For batch incubation, islets of similar sizes (100–150 μm) were handpicked and pre-incubated for 30 min in Krebs-Ringer bicarbonate buffer (KRBB) containing 1 mM glucose and 2 g/l bovine serum albumin (Sigma, St. Louis, MO). Batches of 4 islets were then incubated for 1 h in KRBB buffer containing either 1 or 15 mM glucose. After each incubation, aliquots of incubation medium were collected for measurement of insulin with ELISA (Insulin mouse ELISA; Mercodia, Uppsala, Sweden). For insulin content, islets were extracted in acid ethanol at 4°C.

β-cell mass

Pancreata from 3-4-month old mice were dissected in a standardized fashion and then weighed, fixed overnight in Stefanini's solution (2% paraformaldehyde and 0.2% picric acid in 0.1 M PBS, pH 7.2), washed in Tyrode's solution containing 10% sucrose, and frozen on dry ice. Consecutive 10 μm-thick sections were cut from the tissue and mounted on glass slides. Immunostaining for insulin was performed with guinea pig anti-proinsulin antibodies (dilution 1:5000; EuroDiagnostica, Malmö, Sweden) overnight at 4°C; immune complex were detected with secondary anti-guinea pig antibody conjugated with Texas Red (Jackson Immuno Research Laboratories, PA, USA). The insulin-immunostained area and total section area were measured using Nis-Elements (Nikon, Tokyo, Japan). All islets in 9 sections from 3 parts of the pancreas were analyzed. The immunostained sections were at 200 μm intervals to avoid

measurement of the same islets twice. In total, 583 islets (63-178 islets/group) were measured. 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 14 week old female mice islets, using a procedure described previously with slight modification (Capito and Hedeskov, 1974). Briefly, 25 same-sized islets were picked and washed with 1 ml of cold Hank's balanced salt solution containing 500 µmol/l isobutylmethylxanthine (IBMX). The islets were then quick-frozen in an ethanol/dry ice bath followed by extraction with 95% ethanol. The extracted samples were centrifuged at 20,000 rcf for 30 min and supernatant was subsequently evaporated to dryness. The dried samples were redissolved in 100 µl of assay buffer and used for cAMP determination using cAMP enzyme-linked immunosorbent assay kit (Amersham cAMP biotrak EIA system, GE Healthcare, Buckinghamshire, UK). 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

The population-based PPP-Botnia Study was carried out in three research centers in the Botnia region in western Finland (Isomaa et al., 2010; Lyssenko et al., 2009). For the present study, we invited 30-75 year-old non-diabetic participants, who had two copies of the rs10830963 risk allele (GG, n=240), from the two largest centers. For each GG-carrier, three controls with two copies of the WT allele (CC, n=1059) were matched (center, gender, age ± 3 years, BMI ± 1-2 kg/m^2, glucose tolerance: NGT, IFG, IGT). 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, abnormal creatinine or liver enzymes. Two GG and four CC carriers discontinued the study (dizziness, hospitalization for other causes, panic attack, one declined further contact). The 23 GG and 22 CC carriers who completed the study had similar median (interquartile) age [50.3 (21.4) vs 50.1 (15.4) yrs] and BMI [26.5 (4.5) vs. 27.3 (5.7) kg/m^2] as well as gender distribution (M/F 11/12 vs. 11/11) and proportion with family history of diabetes (N=12/23 vs. 10/22). They received 4 mg of melatonin (Circadin®, Oy Leiras Finland Ab) once daily in the evening for three months. Before commencing treatment with melatonin and after 3 months they participated in a 75g OGTT with measurement of plasma glucose

(Hemocue, Ängelholm, Sweden) and serum insulin (AutoDelfia; Perkin Elmer Finland, Turku, Finland) at 0, 30, 60, 120 min; plasma glucagon (Glucagon RIA, Millipore, St. Charles, Missouri, USA) and serum melatonin (Melatonin Direct RIA, Labor Diagnostika Nord, Nordhorn, Germany) at 0 min. Insulin sensitivity was evaluated using the HOMA insulin resistance index ($HOMA_{IR}$ =fS-insulin * fP-glucose / 22.5) and the insulin sensitivity index (ISI) calculated from the OGTT data as $10,000/\sqrt{(}$fP-glucose x fS-insulin x mean $OGTT_{glucose}$ x mean $OGTT_{insulin}$). β-cell function was assessed as the corrected insulin response (CIR) during the OGTT (100 x $insulin_{30}$/[$glucose_{30}$ x ($glucose_{30}$ – 3.89)]) (Hanson et al., 2000), or a disposition index (DI = ISI x CIR) (Bergman et al., 2002; Matsuda and DeFronzo, 1999). The study protocol was accepted by the Finnish Medicines Agency and the ethical committee of the Pirkanmaa Hospital District and registered in Eudract and Clinical Trials registers (EudraCT-2011-005360-22, NCT01705639).

Sleep quantity and quality

Sleep was objectively measured with actigraphs (Actiwatch AW4 and AW7, Cambridge Neurotechnology Ltd., UK). The devices were worn on the nondominant wrist for an average of 8 nights before the melatonin treatment and on average 7 nights before the second OGTT at three months (Supplemental Table 4). The participants were instructed to maintain a sleep log on bedtimes and waking times, temporary pauses in actigraph registration (e.g., while taking a shower), and significant events that might affect sleep duration or quality (e.g., illness likely to disturb sleep). The participants were instructed to press a button (event marker) in the actigraph at bedtime and waking times. All participants provided completed sleep logs, including written sleep logs and electronic event markers of the bedtimes and waking times. The activity data were visually inspected to detect significant discrepancies among the sleep logs, event markers, and activity patterns. If the same night had several event markers, the most recent was compared with the sleep log. If the sleep log was not synchronous with the event marker, the event marker served to define the bedtime. Nights were excluded from sleep analyses if (a) the actigraph was not in use, (b) information on bedtimes was missing, (c) the data on reported bedtime indicated the participant was already asleep (probably the bedtime was not correctly reported), (d) information on waking time was missing and the activity pattern was unclear, or (e) the participant reported a change in normal life due to, for example, illness or travel.

Data were scored with Actiwatch Activity & Sleep Analysis version 7.38 software with medium sensitivity and a one-minute epoch duration, as recommended by the manufacturer. The scored sleep data for each participant were averaged over the valid registration nights. Sleep duration refers to actual time asleep in hours and minutes. We used the validated

Actiwatch algorithm (Kushida et al., 2001), which defines "Sleep start" as ten minutes of consecutively recorded immobile data, with no more than 1 epoch of movement within that time period. For "Sleep end", the algorithm looks backwards from the last sample in the analysis window for a specific consecutive period (6 minutes) of activity below the threshold (≤ 6 counts) and classifies the last epoch in this period as "Sleep end". Sleep efficiency was defined as actual time asleep divided by time in bed in percent. The wake after sleep onset (WASO) refers to minutes spent awake after sleep onset. Sleep was also self-reported by using two well-validated questionnaires measuring sleep quality, the Pittsburgh Sleep Quality Index (Buysse et al., 1989), and daytime sleepiness, the Epworth Sleepiness Scale (Johns, 1991).

Statistical analysis

Statistical calculations were performed with Graphpad Prism 5 (GraphPad software, San Diego, CA) or SPSS21 (IBM; New Orchard Road, Armonk, New York 10504). 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 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. For the sleep data, ANOVA was used for between group comparisons (baseline data, follow-up data), and repeated-measured ANOVA for within-person comparisons in response to treatment. We also tested for genotype x treatment interactions. Nominal p-values without correction for multiple testing are shown.

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