Melatonin is produced during the night and regulates sleep and circadian rhythms. Loss-of-function variants in MTNR1B, which encodes the melatonin receptor MT$_2$, a G protein–coupled receptor (GPCR), are associated with an increased risk of type 2 diabetes (T2D). To identify specific T2D-associated signaling pathway(s), we profiled the signaling output of 40 MT$_2$ variants by monitoring spontaneous (ligand-independent) and melatonin-induced activation of multiple signaling effectors. Genetic association analysis showed that defects in the melatonin-induced activation of G$_{ai1}$ and G$_{i2}$ proteins and in spontaneous β-arrestin2 recruitment to MT$_2$ were the most statistically significantly associated with an increased T2D risk. Computational variant impact prediction by in silico evolutionary lineage analysis strongly correlated with the measured phenotypic effect of each variant, providing a predictive tool for future studies on GPCR variants. Together, this large-scale functional study provides an operational framework for the postgenomic analysis of the multiple GPCR variants present in the human population. The association of T2D risk with signaling pathway–specific defects opens avenues for pathway-specific personalized therapeutic intervention and reveals the potential relevance of MT$_2$ function during the day, when melatonin is undetectable, but spontaneous activity of the receptor occurs.

INTRODUCTION

Type 2 diabetes mellitus (T2D) is characterized by high blood glucose concentrations in the context of insulin resistance and impaired insulin secretion from pancreatic β cells (1). The prevalence of T2D has increased over the last 20 years and closely parallels the mounting epidemic of obesity. G protein–coupled receptors (GPCRs) are promising drug targets for metabolic diseases, including T2D (2, 3). Agonists of GPCRs, such as the glucagon-like peptide 1 receptor (GLP1R), are clinically used to improve glucose tolerance in T2D patients (4). Furthermore, drugs targeting other GPCRs, such as the gastric inhibitory polypeptide receptor (GIPR), the free fatty acid receptor 1 (FFAR1; also known as GPR40), and the glucose-dependent insulinotropic receptor (GPR119), are currently in clinical trials and at different stages of development for diabetes therapy (3).

In addition to several environmental risk factors, T2D has a strong genetic basis. Genome-wide association studies and their meta-analyses have identified more than 100 loci containing single-nucleotide polymorphisms (SNPs) that are statistically significantly associated with T2D risk and altered glycemic values or insulin secretion (5). The common SNP rs10830963 is located in the unique intron of MTNR1B, which encodes MT$_2$, the melatonin (MLT) receptor, and is statistically significantly associated with increased fasting plasma glucose concentrations, reduced early insulin response to glucose, and increased risk of both T2D and gestational diabetes mellitus (6–8). The homozygous T2D-associated SNP [corresponding to a GG genotype; the wild-type (WT) sequence is CC] is associated with a twofold increase in cis MTNR1B messenger RNA (mRNA) abundance in human pancreatic islets compared with the amount in the islets of CC genotype carriers (7, 9–11), suggesting that increased MLT signaling might be associated with an increased T2D risk. However, when we performed a large-scale exon resequencing of MTNR1B, we found that only rare, nonsynonymous variants with a loss–of-function phenotype strongly associated with increased T2D risk (odds ratio of 5 when analyzed in aggregation) (12). Considering these apparently contradictory findings, the role of MT$_2$ signaling on T2D risk in humans remains unanswered.

MT$_2$ belongs to the MLT receptor family of GPCRs, which is composed of MT$_1$, MT$_2$, and the orphan GPR50 based on their sequence homologies. However, only MT$_1$ and MT$_2$ and not GPR50 bind to MLT and do so with high affinity (13–15). MLT is mainly secreted from the pineal gland in a circadian manner with high plasma concentrations during the night (16). MLT receptors are involved in the regulation of sleep, circadian and seasonal rhythms, immune functions, retinal physiology, and glucose homeostasis (17). How MLT modulates glucose homeostasis in humans is not well understood. In rodent β cell lines, MLT decreases insulin secretion (18, 19), whereas studies of isolated human islets showed that MLT stimulates insulin secretion (20, 21). It is well known that MLT exerts opposing effects on specific functions in a species-dependent manner (22). Epidemiological studies demonstrated that people working on different shifts, compared to day workers, present with a marked dysfunction in nocturnal MLT secretion and a higher risk of cardiovascular disorders, metabolic diseases (including T2D), and cancer. A 12-year prospective study showed...
that decreased nocturnal MLT secretion in nurses working on rotating night shifts is associated with a higher risk of developing T2D (23).

Several sites of MLT action are possible because MT1 and MT2 are present in many central and peripheral tissues, including insulin target tissues, such as liver, muscle, and adipose tissue (19, 24). The amounts of mRNA transcripts for MTNR1A (encoding MT1) and MTNR1B are very low or undetectable in human islets and only detectable in a small subset of β cells (25–27), raising questions about the relevance of human islets as an MLT target tissue (28). In the hypothalamic suprachiasmatic nuclei, MT1 and MT2 regulate the circadian rhythms of the master clock in mammals, and disruption of central and peripheral circadian rhythms leads to metabolic disorders, including T2D (29–31). The T2D-associated SNP rs10830963, which is in an intron unique to MTNR1B, affects the dynamics of MLT secretion (32). Therefore, it is possible that the global dysregulation of MT2 signaling contributes to T2D pathophysiology through the alteration of circadian rhythms.

Given that GPCRs can engage multiple distinct signaling pathways and that biased ligands alter the balance of activated signaling pathways (33, 34), the objective of the current study was to assess the effect of all MTNR1B variants identified by Bonnefond et al. (12) on multiple pathways engaged upon MT2 activation. We generated a “signaling signature” for each variant receptor to investigate whether the association between MT2 loss of function and T2D was linked to specific signaling defects. MT2 has been described to activate three signaling pathways. Through G\(_i\)-inhibiting proteins, MT2 inhibits the production of cyclic adenosine monophosphate (cAMP) and stimulates extracellular signal–regulated kinase 1 and 2 (ERK1/2). To determine whether MT2 can activate these signaling pathways, we performed cAMP assays and ERK1/2 activation assays, respectively. Both G\(_d\)\(_i\) and G\(_e\) inhibit the activity of adenylyl cyclases to inhibit cAMP production; therefore, we assessed the inhibition of forskolin-stimulated cAMP production by MLT-activated MT2. MT2 also displayed spontaneous activity toward both G\(_d\)\(_i\) and G\(_e\), and MLT promoted a concentration-dependent increase in their activity with similar EC\(_{50}\) (half maximal effective concentration) values for both G proteins. The opposite direction of the presented concentration-response curves reflect the different natures of the biosensors used to test their activity, which results in a decrease in BRET for the sensor monitoring the separation of the G\(_d\) subunit from the G\(_7\) subunit (used for G\(_d\)\(_i\)) and the sensor detecting the interaction of the dissociated G\(_e\) with GRK2 (used for G\(_e\)). MT2 also displayed spontaneous activity toward the recruitment of β-arrestin2 in the absence of MLT, an activity that was concentration-dependently increased by the addition of MLT (fig. S1E).

To confirm signaling downstream of G proteins and β-arrestin2, we performed cAMP assays and ERK1/2 activation assays, respectively. Both G\(_d\)\(_i\) and G\(_e\) inhibit the activity of adenylyl cyclases to inhibit cAMP production; therefore, we assessed the inhibition of forskolin-stimulated cAMP production by MLT-activated MT2. MLT triggered a dose-dependent reduction in cAMP concentration with maximal inhibition of about 30%, consistent with the coupling of MT2 to G proteins that inhibit adenylyl cyclase activity (fig. S1F). β-Arrestins promote receptor internalization and desensitization of G protein–dependent signaling at receptors located at the plasma membrane (37). In addition, β-arrestins function as independent signaling transmitters (38) or mediate sustained endosomal signaling (39–42). Both G protein–mediated and β-arrestin–mediated signaling can activate ERK1/2. To determine whether MT2 can stimulate ERK activation, we performed an ERK activation assay (43) and found that MLT stimulated a dose-dependent increase in ERK activation in MT2-expressing cells (fig. S1G).

Evaluation of spontaneous G\(_d\)\(_i\) and G\(_e\) signaling and β-arrestin2 recruitment by MT2 variants

Subsequently, we monitored the effects of the 40 nonsynonymous MT2 variants (including 38 rare and the 2 common p.G24E and p.K243R variants), which are distributed throughout the receptor (see fig. S2 for variant positions in the receptor), on the direct activation of G\(_d\)\(_i\) and G\(_e\) proteins, downstream inhibition of forskolin-stimulated cAMP production, and the recruitment of β-arrestin2, and ERK1/2 activation. To control for the potential influence of different cell surface abundances on signaling activity, we assessed cell surface receptor abundance by enzyme-linked immunosorbent assay (ELISA) for all tested forms of the variants and WT receptors (fig. S3). Four mutants are classified as loss-of-MLT binding (p.A42P, p.L60R, p.P95L, and p.Y308S) on the basis of results obtained in a radioligand binding assay (12). We measured the spontaneous (agonist-independent) activation of G\(_d\)\(_i\) and G\(_e\) by the MT2 variants. For WT MT2, the spontaneous activity represented 51.3 and 22.5% of the maximal MLT-stimulated activity for G\(_d\)\(_i\) and G\(_e\), respectively (fig. S1, C and D). Seventeen MT2 variants displayed reduced spontaneous activation.

We also determined the spontaneous recruitment of β-arrestin2 to the MT2 variants. Similar to G protein activation, we observed high spontaneous β-arrestin2 recruitment for WT MT2, representing 70% of the maximal response (fig. S1E). Spontaneous β-arrestin2 recruitment was statistically significantly reduced for 15 variants (Fig. 1C and table S3). The four mutants that exhibit loss-of-MLT binding (p.A42P, p.L60R, p.P95L, and p.Y308S) did not display any spontaneous β-arrestin2 recruitment, confirming that the spontaneous β-arrestin2 recruitment monitored for the WT MT2 was receptor-dependent and does not represent an intrinsic feature of the assay. None of the variants showed increased spontaneous β-arrestin2 recruitment (Fig. 1C), suggesting that the constraints that restrict constitutive activation of G proteins are different from those controlling spontaneous β-arrestin recruitment.

**Evaluation of agonist-induced Gα11 and Gα2 signaling by MT2 variants**

The effect of MLT on Gα11 or Gα2 activation was also differentially altered by the variants. For each variant, full concentration-response curves were performed, and both the EC50 (potency) and the maximal agonist-mediated response (efficacy; Emax) were determined (Fig. 2, fig. S4, and tables S1 and S2). From this analysis, we grouped the variants into eight categories (Fig. 2 and fig. S4): those that were previously classified as loss-of-MLT binding variants (Fig. 2A and fig. S4A), those that exhibited impaired signaling through all pathways (Fig. 2B and fig. S4B), those that exhibited impaired signaling in two or more pathways (Fig. 2C and fig. S4C), those with specific defects in MLT-stimulated G protein–specific signaling (Fig. 2D and fig. S4D), those with a specific defect in β-arrestin recruitment (Fig. 2E and fig. S4E), those with a specific defect in ERK activation (Fig. 2F), those that exhibited a gain of function (Fig. 2G), and those that were similar to WT MT2 (fig. S4F).

Evaluation of ERK signaling by MT2 variants

ERK phosphorylation upon MLT stimulation was used as a proxy for activation. For WT MT2, ERK activation was fully blocked by the Gαi1 inhibitor pertussis toxin and was unaffected by RNAi (RNA interference)–mediated silencing of β-arrestin1 and β-arrestin2 (fig. S5), indicating that ERK activation occurred downstream of G protein activation. All MT2 variants that were impaired for both the Gαi/cAMP and β-arrestin2 pathways also exhibited reduced phosphorylation of ERK in response to MLT (Fig. 2, fig. S4, and table S5). Other variants showed partially impaired ERK activation (p.A74T, p.V124I, and p.T201M) together with partial impairment of both G protein–mediated signaling and β-arrestin2 recruitment or with a specific Gαi1 signaling defect (p.R154H) or with a specific Gαz signaling defect (p.M120I). Furthermore, the ERK signaling–defective variant p.M146V was the only variant with normal Gαi/cAMP signaling and β-arrestin2 recruitment, suggesting the potential involvement of additional pathway(s) in mediating MT2–dependent ERK activation. The p.A359E variant differed from all of the others: It exhibited a substantially increased ERK response (246% Emax of the WT) (table S5), a property that extended to a lesser extent to Gαi1 activation and β-arrestin2 recruitment (Fig. 2).

Evaluation of the relationship among signaling alterations exhibited by the MT2 variants

To determine whether there was a link between the effect of the mutation on spontaneous or agonist-stimulated receptor activities, correlations between the effects on the two parameters were performed for the different pathways (Fig. 3A and fig. S6, A and B). In addition, to assess the possible functional links between two pathways, correlation between the effect on each pathway was performed in a pairwise manner (Fig. 3, B and C). We also performed pairwise comparisons of spontaneous activity (fig. S6, C to E). Spontaneous Gαi1 and Gαz activation only modestly correlated with the corresponding MLT-induced activation of Gαi1 (Fig. 3A, left) and Gαz (Fig. 3A, middle). A typical example is the p.M120V variant, which exhibited markedly reduced spontaneous Gαi1 activity and had WT–like agonist-stimulated activity (Fig. 3A, left). Similar to the low correlation between spontaneous and agonist-stimulated G protein activation, the correlation between spontaneous and MLT–induced β-arrestin2 recruitment was also low (Fig. 3A, right). This lack of correlation between spontaneous and agonist-induced activity indicated that the modes of spontaneous and MLT-induced receptor activity were distinct with potentially different allosteric constraints. In contrast, there was a strong correlation between the spontaneous activation of Gαi1 and Gαz (fig. S5C) and between the MLT–induced activation of Gαi1 and Gαz (Fig. 3C, Gαi1 versus Gαz). These correlations suggest that similar receptor-mediated activation modes control these two Gαi family members.

To test for a link between G protein– and β-arrestin2–dependent events, we correlated MLT–induced Gαi1 and Gαz activation and cAMP inhibition with MLT–induced β-arrestin2 recruitment (Fig. 3B). We also tested the correlation between MLT–induced G protein activation and spontaneous β-arrestin2 recruitment (fig. S5, A and B) and between spontaneous G protein activation and spontaneous β-arrestin2 recruitment (fig. S5, C to E). All showed moderate, yet statistically significant correlations, which contrasted with the tightly correlated G protein–dependent events (Fig. 3C), indicating that G protein activation and β-arrestin recruitment rely on at least partially independent processes. Together, these data reveal a general trend of reduced spontaneous and MLT–induced activity for MT2 mutants at the level of β-arrestin2 recruitment and
Spontaneous activity versus MLT-induced effect

Fig. 3. Relationships between spontaneous and MLT-induced MT2 variant activation, between G protein- and β-арrestин2–dependent events, and between different G protein signaling events. (A) Correlation plots between the spontaneous and MLT-induced effects (E_{max}) of each variant MT2 for Gα_{i1} activation (left), Gα_{z} activation (middle), and β-арrestин2 recruitment (right). (B) Correlation plots between the E_{max} values of MLT-induced Gα_{i1} activation, Gα_{z} activation, and cAMP inhibition with β-арrestин2 recruitment for each variant MT2. (C) Correlation plots between the E_{max} values of MLT-induced Gα_{i1} activation and Gα_{z} activation (left), Gα_{i1} activation and cAMP inhibition of each variant MT2. Spontaneous activities and E_{max} values represent means ± SEM of at least three experiments and were normalized to those of the WT MT2. Data were fitted by linear regression analysis, and R^2 values were obtained for the overall correlation. All R values are statistically significant with P values <0.0001. See also fig. S6 for correlations between spontaneous and agonist-promoted activity for G proteins and β-арестин signaling parameters.
G protein activation and highlight the independent nature of these two processes.

**Generation of signaling signatures of MT2 variants**

To represent the signaling characteristics of the different variants, the overall signaling efficiencies of the WT and mutant receptors were assessed for each outcome using the operational model of Black and Leff (44) as modified by Kenakin et al. (45). The transduction coefficient \( \log(t/K_a) \) was used as an indication of the signaling efficiency of each variant, and the comparison between WT receptor and each of the variants was then obtained by subtracting the transduction coefficient \( \log(t/K_a) \) of the mutants from that of the WT and expressed as \( \Delta \log(t/K_a) \) (tables S1 to S5). We plotted radial graphs of the calculated \( \Delta \log(t/K_a) \) together with the spontaneous and MLT-induced signaling efficacy to represent a signaling signature for each MT2 variant. This representation revealed several groups of variants with distinctive signaling signatures (Fig. 4). For example, 12 variants have strong defects in the five signaling cascades considered (p.A42P, p.L60R, p.P95L, p.S123R, P.R138C/H/L, p.R222H, p.I223T, p.F250V, p.Y308S, and p.R316H), 6 variants showed specific defects in β-arrestin2 recruitment (p.L166I, p.R231H, p.E237K, p.S238G, p.K243R, and p.D246N), and 3 variants displayed G protein–specific defects (p.R154H, p.Y141F, and p.R330W). Three variants (p.M120I, p.P36S, and p.A359E) resulted in increased signaling activity compared to that of WT MT2 in response to MLT.

**Evolutionary action analysis of MT2 variants**

The large amount of functional data collected here provides a tool to validate in silico models predicting variant receptor functionality. One of these models, evolutionary action (EA) (36) predicts the effect of a variant on protein function using the relative importance of each residue based on evolutionary divergence, evolutionary trace (46), combined with the likelihood to observe a given amino acid substitution for another across evolution measured as the substitution log odds (36). On the basis of these phylogenetic patterns of variation, an EA score from 0 (benign) to 100 (highly detrimental or impactful) was assigned for each MT2 variant (Fig. 5A). To assess whether these empirical EA scores could be predictive of the functional impact observed in the signaling assays, we assigned a “phenotype score” to each variant that was calculated by averaging the 13 values corresponding to the normalized functional parameters used for the signaling signatures. The predictive EA scores correlated with the experimentally determined phenotype scores (Fig. 5B), indicating that the degree of measured functional divergence of the variants from the WT correlated with the degree of predicted evolutionary divergence. Overall, the good correlation of phenotypic scores with EA scores confirmed the robustness of evolutionary action in predicting the effect of variants on receptor fitness.

**Association between functional MT2 defects and T2D risk**

On the basis of the different signaling signatures observed for the MT2 variants, they clustered into nine groups. For each of these nine groups, the association between T2D risk and the aggregation of the variants was assessed by the kernel-based adaptive cluster method (47) as previously described (Table 1) (12). By applying Bonferroni correction, we considered \( P < 0.0056 \) to be statistically significant, and we considered \( P \) values between 0.05 and 0.0056 to be a trend of association. We found that rare variants with defects in MLT-induced activation of \( \text{G}_{\alpha_1} \) or \( \text{G}_{\alpha_5} \) in spontaneous β-arrestin2 recruitment were the most strongly and statistically significantly associated with increased T2D risk. For example, rare variants with defects in MLT-induced activation of \( \text{G}_{\alpha_1} \) had an odds ratio of 3.25 with a 95% confidence interval of 1.73 to 6.10 and a \( P \) value of \( 2.4 \times 10^{-4} \) (Table 1).

In contrast, all of the other groups characterized by different signaling defects showed only a trend of association with T2D. The absence of an association of defective MLT-induced β-arrestin2 recruitment with T2D is consistent with the observation that the frequent p.K243R variant (rs61747139), which exhibits defective MLT-induced β-arrestin2 recruitment, is not associated with T2D (12, 48). Furthermore, the “neutral” rare variants were not found to be associated with T2D risk (Table 1). This is consistent with lack of risk association between T2D and the common p.G24E variant (rs8192552) (12, 48), which is also neutral.

**DISCUSSION**

Our extensive functional genomics study on 40 rare or very rare MT2 variants identified an overall loss-of-function phenotype for many parameters and pathways measured. The observed disconnection of spontaneous versus MLT-induced activity as well as G protein–versus β-arrestin–dependent signaling of these variants enabled us to dissect the respective contributions of each of these parameters and to reveal the strongest associations to increased T2D risk with defective MLT-induced \( \text{G}_{\alpha_1} \) and \( \text{G}_{\alpha_5} \) activation and defective spontaneous β-arrestin2 recruitment.

**Connecting rare GPCR variants with common diseases: A general framework**

The present study provides a framework to correlate alterations in specific signaling parameters with the risk to develop a common disease. Our study is distinct because the functional basis or relevance of most genetic risk factors remains either unknown or is restricted to a simple loss- and gain-of-function phenotype classification. It is now well established that the intracellular signaling of GPCRs is highly interconnected, involving multiple signaling pathways. Defining the full functional profile or signaling signature of a GPCR and its variants is important to understand the functional defect linked to disease-associated receptor variants. Whereas the number of such GPCR variants has been largely underestimated because of their low frequency, systematic whole-genome sequencing projects revealed a large interindividual diversity when considering large populations, a relevant scenario in the case of common diseases like T2D.

For genes coding for non-odorant GPCRs in humans, an average of 32 nonsynonymous variants have been estimated to exist for each GPCR in a sample of 10,000 individuals (49, 50). This estimation provides a first measure of the number of rare GPCR variants in the human population and gives an idea of the future challenges in terms of their functional characterization to understand their effect on diseases. In terms of the number of variants, the 40 variants identified in the MTNR1B gene in a cohort of ~10,000 individuals (12) seem to fit with this assumption. As demonstrated by our study, such a number of variants can be sufficient to identify receptor defects at the level of individual signaling pathways. Application of the operational model of Kenakin and Christopoulos (34, 45), originally designed to compare the relative signaling efficiency of different ligands, was very instructive in defining an integrative measure for signaling defects that went beyond the determination of the isolated signaling parameters. This is exemplified by the p.R154H variant for which defects in \( \text{G}_{\alpha_1} \) activation only became visible at the level of the
Fig. 4. Graphical representation of the signaling signatures of MT2 variants. Radial graphs representing spontaneous and MLT-induced activation of Ga11 and Ga20, inhibition of cAMP production, recruitment of β-arrestin2, and activation of ERK by WT MT2 and the 40 indicated MT2 variants. The activity of WT MT2 was set as zero. Values of variants with enhanced properties ranged from 0 to +1, and those with impaired properties ranged from 0 to −1. The scale of all radial graphs ranged from −1 to +1. S, spontaneous; Em, agonist-mediated efficacy; Δ, Δlog(s/Kd). See also tables S1 to S5 for complete data sets for spontaneous and agonist-mediated signaling activity of the MT2 variants.
Given that GPCRs, such as MT2, can signal through various cascades with T2D risk and defects of every MT2 variant were defined by phenotype score and correlated 

EA score of MT2 variants and the corresponding experimentally de

cated MT2 variant. EA ranges from 0 to 100, with a score of 0 predicted as benign 

An evolutionary action score was calculated for each indicated MT2 variant. EA ranges from 0 to 100, with a score of 0 predicted as benign and a score of 100 predicted as highly impactful or detrimental to protein function. Scores are colored on the basis of EA [EA = 0 (pink) to EA = 100 (blue)]. (B) Functional defects of every MT2 variant were defined by phenotype score and correlated with the EA score. Data were fitted by linear regression analysis, and $R^2$ values were obtained for the overall correlation. Correlation value was statistically significant, $P < 0.0001$. The A42P MT2 variant was excluded from the $R^2$ calculation because of the known detrimental effect of proline residues on general protein structure. See also tables S1 to S5 for complete data sets for spontaneous and agonist-mediated signaling activity of the MT2 variants.

$\Delta \log(\tau/K_A)$ transduction ratio because it integrates both potencies and maximal efficacies. A decrease in the maximal agonist-mediated efficacy and a rightward logEC$_{50}$ shift were observed for this variant but were not statistically significant. However, when both maximal efficacy and potency were integrated, the global response depicted by $\Delta \log(\tau/K_A)$ value was statistically significantly reduced compared to that of the WT receptor. A good correlation was seen between the EA score of MT2 variants and the corresponding experimentally determined phenotypic scores, which indicates the usefulness of the EA score to predict the functional effect of a specific GPCR variant. The EA score might therefore be applied to obtain a first classification of GPCR variants before entering into large-scale functional studies. Together, our data provide an operational template for the evaluation of the functional effect of GPCR variants on common traits and diseases.

### Specific pathway parameters of MT2 variants associated with T2D risk

Given that GPCRs, such as MT2, can signal through various cascades and that different variants have the potential to modulate one or more signaling parameters, a complete functional characterization is required to understand the full effect of receptor variants on disease risk. Here, we determined both spontaneous and MLT-induced MT2 activity for five signaling events (G$_{i1}$ and G$_{z}$ activation, inhibition of cAMP production, $\beta$-arrestin2 recruitment, and ERK1/2 activation). Ten of 38 rare receptor variants had a phenotype that was indistinguishable from that of the WT receptor and were therefore classified as neutral variants. The 28 other variants showed functional defects compared to the WT receptor. Only the variant p.A359E showed a robust gain-of-function phenotype with 246, 145, and 130% increases in the $E_{\text{max}}$ values of ERK activation, G$_{i1}$ activation, and $\beta$-arrestin2 recruitment, respectively. It would be interesting in a follow-up study to determine the effect of this increased MT2 function on an individual’s metabolism. Together, our data show that loss-of-function MT2 variants at different functional levels are associated with increased T2D risk. Detailed genetic association analysis detected three parameters that stand out in terms of the statistical significance of association with T2D risk: MLT-induced activation of G$_{i1}$ (19 variants) and G$_{z}$ (22 variants) and spontaneous $\beta$-arrestin2 recruitment (15 variants). The independence of signaling events associated with G protein activation and $\beta$-arrestin2 recruitment was further highlighted by the poor correlation between them for the 40 MT2 variants. The importance of G$_{i1}$ activation underlines the general importance of this well-characterized pathway for MT2. The activation of G$_{z}$ by MT2 has not been reported previously, but the importance of impaired G$_{z}$ coupling for the association with T2D risk is of interest because the tissue distribution and GPCR coupling profile of G$_{z}$ are more restricted than those of G$_{i1}$. G$_{z}$ is mainly expressed in different brain regions and in retinal ganglion cells (51, 52), all regions for which MLT receptor expression has been reported (53, 54). In addition, the intrinsic GTPase (guanosine triphosphatase) activity of G$_{z}$ is very slow, with a hydrolysis rate that is 200-fold slower than that determined for any other Gz proteins (55). These results introduce G$_{z}$ as a previously uncharacterized player in MT2 signaling with a potential effect on T2D risk.

Although the recruitment of $\beta$-arrestin2 to MT2 in transfected cells has been reported (56, 57), the physiological importance of this phenomenon and its robustness in a cellular context expressing endogenous MT2 receptors remain unclear. MT2 has indeed a poor capacity for MLT-induced $\beta$-arrestin recruitment, an observation that was confirmed here. Part of this difficulty to reveal ligand-induced $\beta$-arrestin2 recruitment might be explained by the high degree of spontaneous $\beta$-arrestin2 recruitment observed here. This spontaneous activity was completely lost for several variants, including the four loss-of-MLT binding mutants, which suggests that the measured spontaneous activity is receptor-dependent. $\beta$-Arrestins are important regulators of GPCR function and scaffolding platforms for many signaling complexes (38), but the role of $\beta$-arrestin recruitment in MT2 function in peripheral and central tissues is currently unknown. Our results indicating an association between the spontaneous recruitment of $\beta$-arrestin2 to the receptor and T2D risk point to a possible important role of $\beta$-arrestin2 recruitment to MT2.

Another unexpected and potentially relevant outcome of our study is the association of spontaneous MT2 activity, namely, $\beta$-arrestin2 recruitment, with T2D risk. The notion that spontaneous and ligand-induced receptor activation of the 40 variants should be considered as independent signaling properties is supported by the poor correlation of these two parameters for three signaling pathways (G$_{i1}$ activation, G$_{z}$ activation, and $\beta$-arrestin2 recruitment), which enabled us to determine the effects of both parameters independently on disease risk. Spontaneous MLT receptor activity was previously described for
Table 1. Association of specific functional defects of rare MT\(_2\) variants with T2D risk. Only rare MT\(_2\) variants (with a frequency <1%) were included. By applying Bonferroni correction, a statistically significant \(P\) value was <0.0056. A \(P\) value between 0.05 and 0.0056 was considered a trend of association (a nominal association). Nine independent association tests were performed on the basis of a T2D case-control study including 2186 individuals with T2D and 4804 controls. CI, confidence interval.

<table>
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<th>Consequences on MT(_2) function</th>
<th>Rare MT(_2) Variants</th>
<th>Frequency (cases)</th>
<th>Frequency (controls)</th>
<th>Odds ratio (95% CI)</th>
<th>(P) value</th>
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<td>Defects in (G_{\alpha_i}) activation</td>
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<td>A2ST, A42P, L60R, P95L, M1200/V, S123R, V124I, R138C/H/L, Y141F, T201M, I223T, F250V, Y308S, R316H</td>
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<td>2.32 (1.17–4.60)</td>
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<td></td>
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<td>A42P, L60R, A74T, P95L, S123R, V124I, R138C/H/L, Y141F, R154H, T201M, R222H, I223T, F250V, Y308S, R316H, R330W, A342V</td>
<td>1.4%</td>
<td>0.7%</td>
<td>3.25 (1.73–6.10)</td>
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<tr>
<td>Defects in (G_{\alpha_x}) activation</td>
<td>Spontaneous</td>
<td>A42P, L60R, P95L, M1200/V, S123R, V124I, R138C/H/L, Y141F, R154H, T201M, R222H, I223T, F250V, Y308S, R316H</td>
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<td>0.5%</td>
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<td>1.4%</td>
<td>0.8%</td>
<td>2.93 (1.57–5.45)</td>
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<td>0.9%</td>
<td>0.6%</td>
<td>2.83 (1.31–6.12)</td>
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<td>Defects in (\beta)-arrestin2 recruitment</td>
<td>Spontaneous</td>
<td>A42P, L60R, P95L, G109A, M1200/V, S123R, R138C/H/L, I223T, F250V, Y308S, A342V</td>
<td>1.3%</td>
<td>0.7%</td>
<td>3.06 (1.59–5.90)</td>
</tr>
<tr>
<td></td>
<td>MLT-induced</td>
<td>A42P, L60R, A74T, P95L, G109A, S123R, V124I, R138C/H/L, Y141F, R154H, R222H, I223T, F250V, Y308S, A342V</td>
<td>2.7%</td>
<td>2.3%</td>
<td>2.13 (1.23–3.70)</td>
</tr>
<tr>
<td>Defects in ERK activation</td>
<td>MLT-induced</td>
<td>A42P, L60R, A74T, P95L, M1200/V, S123R, V124I, R138C/H/L, Y141F, T201M, R222H, I223T, F250V, Y308S, R316H</td>
<td>1.2%</td>
<td>0.8%</td>
<td>2.49 (1.29–4.80)</td>
</tr>
<tr>
<td>Neutral variants</td>
<td></td>
<td>A8S, A13V, G21S, W22L, A25T, P36S, A52T, A234T, I535T, A359E</td>
<td>0.2%</td>
<td>0.2%</td>
<td>2.50 (0.59–10.59)</td>
</tr>
</tbody>
</table>
G proteins in transfected cells and tissues (58–60). Our study shows that MT2 also spontaneously recruits β-arrestin. The importance of constitutive GPCR activity in disease has been discussed in the mesolimbic dopamine system for cannabinoid 1, 5-HT2C, and μ-opioid receptors (61). Spontaneous activity as a risk factor for T2D is particularly interesting in the case of MT2, given that its cognate ligand, MLT, is only produced during the night and absent during the daytime because of a direct inhibitory effect of light on MLT synthesis (62). This opens the interesting possibility that MT2 function might be physiologically relevant not only during the night but also during the day, at a time when the regulation of plasma insulin concentrations is most important in humans.

Whereas the link between the MTNR1B gene and T2D risk has been firmly established in humans (8), the molecular mechanism and relevant MLT target tissues in humans remain largely unknown. Studies on rodent pancreatic β cells indicate that these cells are relevant MLT targets in which MT1 or MT2 activation inhibits insulin secretion through the Gβγ/cAMP pathway (63). However, studies conducted with human pancreatic β cells did not have clear conclusions, and several studies using single-cell transcriptome profiling and RNA sequencing detected MTNR1B transcripts only in a minor fraction (5%) of human islet cells and at extremely low concentrations (27, 64), questioning the physiological relevance and effect of MTNR1B expression in the human pancreas. Functional studies on the common SNP rs10830963, located in the intron of MTNR1B, showed a twofold increase in MTNR1B mRNA abundance in human pancreatic islets of GG T2D risk allele carriers (9). However, the functional importance of this observation remains unclear because increased amounts of mRNA transcripts do not necessarily result in increased numbers of receptors. Furthermore, even if an increase in receptor number occurs, this does not necessarily result in an increased signaling capacity because of the presence of spare receptors or chronic receptor desensitization.

Together with the currently available data, the initially proposed inhibitory action of MT2 in human β cells warrants revision and may be too simplistic. Other potential physiological outputs and MLT target tissues should be considered. Among those are the well-documented effect of MLT on the circadian rhythm of the hypothalamic master clock and the effects on insulin target tissues, such as the adipose tissue (17). Without having clarified this important point, validation of our results obtained in the human embryonic kidney–293 (HEK 293) model system into a physiologically relevant context remains challenging. Our results provide hints about the most relevant signaling pathways potentially involved, which are the activation of Gαi and Gαs and the recruitment of β-arrestin2. These results will direct future research for the relevant physiological outputs of MLT action. The finding that spontaneous receptor activity also contributes to T2D will be important in guiding future studies in humans by also considering the effects of MT2 during the daytime, when MLT is not produced.

In terms of therapeutic perspectives, both medical intervention and lifestyle recommendations can be envisioned. The predominant defects observed in MT2 variants are decreased Emax values, suggesting that improving the residual responsiveness in these patients is a possible therapeutic strategy. Because most of the loss-of-function mutants were defective for G protein activation and β-arrestin2 recruitment, an optimal treatment should aim to improve both components. In terms of lifestyle recommendations, future studies will have to establish whether carriers of MT2 variants suffer from MLT-specific dysfunctions, such as sleep problems or circadian misalignment of sleep-wake cycles with feeding rhythms. If this is the case, carriers of MT2 variants could benefit from specific lifestyle recommendations to decrease their T2D risk. Because carriers of MT2 variants are rare or very rare, such interventions should be seen in the context of personalized medicine. On the basis of the signaling signatures established in our study, patient groups might be identified that would benefit from a similar treatment. Similar examples of other GPCRs are melanocortin MC4 receptor mutants, which are associated with severe early-onset obesity for which defective cell surface expression can be rescued with pharmacological chaperones (65), and gain-of-function mutants of the chemokine receptor CXCR4 identified in patients with the Warts, Hypogammaglobulinemia, Immunodeficiency, and Myelokathexis syndrome (WHIM) syndrome for which the CXCR4 antagonist AMD3100 is currently in clinical trials (66).

Our study further highlights an often-neglected issue, which is the time of blood sampling or functional examination of patients to obtain meaningful results when trying to understand the biology of the MLT system. Apart from the circadian secretion profile of MLT, the constitutive MT2 activity and its potential importance during the day time, when MLT concentrations are low, should also be taken into account in this respect. Finally, the set of 40 natural MT2 variants will constitute a rich source for future structure-function studies of biased receptor signaling.

Conclusion
We provide here a comprehensive framework for the functional characterization of GPCR variants that can serve as a template for future studies on further GPCRs. Our results on rare MT2 variants demonstrate the association of the loss-of-function phenotype with T2D risk, which served as a basis for the dissection of the specific signaling events most statistically significantly associated with the disease. The two main signaling modules of GPCRs, mediated by G proteins and β-arrestins, both seem to contribute to disease risk. Our finding of the association of T2D risk with spontaneous MT2 activity should provide insights with respect to circadian timing, an important aspect of MLT research. The information on disease-associated signaling pathways will be valuable for future studies in defining the still unknown functionally relevant outputs linking MT2 to T2D risk. This information will guide future drug-screening programs intended to improve MT2 function.

MATERIALS AND METHODS
Plasmids and reagents
The 6mycMT2/pcDNA5/FRT plasmids encoding WT MTNR1B and the 40 MTNR1B variants were previously described (12). The 3mycMT2/ARMS2/ProLink (PL) plasmids encoding WT MTNR1B and its variants were generated by subcloning the coding regions of MTNR1B from the 6mycMT2/pcDNA5/FRT plasmids into the pCMV–PL expression vector, pCMV–ARMS2–PK2 (DiscoverX). The resulting plasmid encoded in the C terminus the PL, a small enzyme donor fragment of β-galactosidase (β-gal) complementary peptide, fused to the target receptor. All constructs were verified by Sanger sequencing (Institut Cochin Sequencing Facility). MLT was obtained from Tocris Biosciences.

Cell culture and transfection
HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin.
HEK 293 cells stably expressing a β-arrestin2–β-gal–enzyme acceptor fusion protein (DiscoverX) were maintained in the same culture conditions, but with the addition of hygromycin B to maintain expression of the transgene. All cells were transfected with PEI (polyethylenimine) (Polysciences Inc.) or X-tremeGENE 9 (Roche Diagnostics) according to the manufacturer’s instructions.

Analysis of cell surface receptor expression
ELISAs were performed as previously described (67) using the mouse anti-myc antibody and a horseradish peroxidase–conjugated mouse immunoglobulin G (IgG) whole-sheep secondary antibody (Sigma).

BRET assay
Gαzi activation was assessed by measuring BRET between RlucII–Gαzi and GFP10–Gγ2 upon treatment of HEK 293 cells cotransfected to express Gβ1, receptor, and BRET sensors with MLT (68). This BRET pair was also used for the screening of Gα proteins activated by MT2, except for RlucII–Gαq, RlucII–Gαs, RlucII–Gαq, and RlucII–Gα12/13. For these RlucII–tagged sensors, GFP10–Gγ2 was used given that optimal responsive window is generally obtained using these BRET sensor pairs for these Gα proteins (fig. S1A). For this Gα protein screening, vasopressin receptor type 2 was used as a positive control for Gαs activation, whereas muscarinic type 2, angiotensin type 1, and TPAR receptors were used as positive controls for Gα12/13, Gαq, and Gα12/13, respectively. For Gαzi activation, the MLT–induced increase in BRET between RlucII–Gγ5 and GRK2–FP10 was monitored in the presence of Gαzi, Gβ1, and the receptor. The same BRET pair was also used for the screening of Gα proteins activated by MT2 (fig. S1B). Transfected cells were plated in poly-D-lysine–pretreated 96-well white microplates (100 µl per well; Greiner). Forty-eight hours after transfection, the cells were washed with Dulbecco’s phosphate-buffered saline, and then Tyrode’s buffer was added. After the addition of agonist, the cells were incubated for 10 min at 37°C, and coleterazine 400a (2.5 µM; NanoLight Technology) was added 5 min before BRET reading in a Synergy Neo Microplate reader (BioTek) (acceptor filter: 515 ± 30 nm; donor filter: 410 ± 80 nm). Net BRET represents the BRET signal subtracted by the BRET monitored in the absence of a GFP10–tagged sensor. ΔBRET refers to the difference in net BRET recorded from cells treated with agonist and cells treated with vehicle. For the agonist dose–response curves, the percentage of the response of the WT receptor was calculated from the ΔBRET value obtained from a given variant divided by the ΔBRET obtained from the WT receptor in the same experiment. Spontaneous Gαzi or Gαs activation was calculated from the variant ΔBRET value divided by the ΔBRET obtained from the WT receptor in the same experiment, where ABRET here represents the difference in basal net BRET (vehicle only) in cells expressing biosensors and the receptor and the basal net BRET monitored in cells expressing biosensors only (mock).

Homogeneous time-resolved fluorescence–based cAMP assay
The intracellular concentrations of cAMP were determined in cells transiently expressing MT2 or one of the 40 MT2 variants in the presence of 2 µM forskolin and after 30 min of stimulation with MLT at room temperature by HTRF (homogeneous time-resolved fluorescence) using the “cAMP femto2” Kit (Cisbio) according to the manufacturer’s instructions.

Analysis of β-arrestin2 recruitment
The PathHunter assay (DiscoverX) was used to measure interactions between MT2 and β-arrestin2 as previously described (43). HEK 293 cells stably expressing a fusion protein of β-arrestin2 with a larger fragment of β-gal, called enzyme acceptor, were transiently transfected with the 3mycMT2/PL plasmids. Two days after transfection, the medium was replaced with AssayComplete Cell Plating 4 Reagent (DiscoverX) containing MLT, and the cells were incubated for 2 hours at 37°C. Activation of the MT2/PL stimulates the binding of β-arrestin2–enzyme acceptor fusion protein to the PL–tagged MT2 and forces complementation of the two β-gal enzyme fragments, resulting in the formation of an active β-gal enzyme. This interaction leads to an increase in enzyme activity that can be measured with chemiluminescent PathHunter Detection reagents (DiscoverX; www.discoverx.com/arrestin) and represents β-arrestin2 recruitment to the receptor. Luminescence signal was determined after 60 min of incubation at room temperature.

Measurement of pERK1/2 abundance
Intracellular pERK1/2 was measured using the SureFire pERK Kit (Thry-Tyr202,PerkinElmer) in HEK 293T cells transiently expressing the MTNR1B or the MTNR1B variants, as previously described (69). Given that the peak of pERK phosphorylation was previously observed for MT2 at 5 min after MLT stimulation (12), this time point was chosen to test the ability of MT2 variants to activate ERK in MLT dose-response curves.

Evolutionary action analysis
The predicted effect of each variant within the MT2 receptor was calculated, as previously described, (36) using the amino acid sequence deduced from the MTNR1B gene for the query.

Statistical analysis
LogEC50, logIC50 (half maximal inhibitory concentration), and agonist-induced Emax values were determined by nonlinear regression with a variable Hill slope using GraphPad Prism software (version 6.0). For each assay, the agonist-induced Emax value for every MT2 variant was normalized as a percentage of the maximal MLT–stimulated response of the WT receptor (set at 100) monitored in parallel with the receptor variant. The dose-response curves were fitted to an operational model of agonism designed by Kenakin and Christopholus (34, 45) to obtain log(τ/KA) values for the WT receptor and its variants. Normally, an agonist is set as a reference agonist, against which within–pathway comparisons for the same receptor to other agonists can be made and expressed as Δlog(τ/KA). Here, within–pathway comparisons were made between MT2 variants and the WT receptor. For each variant, the spontaneous activation of Gαzi, and of Gαs, and spontaneous recruitment of β-arrestin2 activation were expressed as a percentage of that of the WT receptor. Normalized differences were calculated on values corresponding to the spontaneous activation, agonist–induced Emax, and Δlog(τ/KA) values using the following formula: (variant − WT)/(variant + WT). In the case of Δlog(τ/KA), before normalization, the anti–logs were first calculated and then were fitted to the aforementioned formula. Positive and negative values represent mutations with better or worse responses, respectively, than those of the WT receptor. Subsequently, logIC50, logEC50, agonist–induced Emax, and Δlog(τ/KA) values were expressed as means ± SEM of the indicated number of experiments (n). Statistical analysis for logIC50, logEC50, and agonist–induced Emax was
performed by comparing independent fits with a global fit that shared the selected parameter, whereas a one-sample t test was used to examine the mean differences between WT MT2 and its variants for the values corresponding to the spontaneous activation and Alog(τ/Kᵦ) ratios.

**T2D case-control association study**

As previously described (12), rare MTNR1B variants (with a frequency <1%) were analyzed in aggregation according to their functional effect. In a T2D case-control study including 2186 individuals with T2D and 4804 controls with normal fasting glucose concentrations, all cohort studies followed ethical principles defined in genome-wide association studies for type 2 diabetes and glycosylated traits to their downstream effectors. PLOS Genet. 11, e1005694 (2015).


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Type 2 diabetes–associated variants of the MT₂ melatonin receptor affect distinct modes of signaling

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Melatonin meets diabetes

Some of the single-nucleotide polymorphisms associated with type 2 diabetes (T2D) occur in the gene encoding the melatonin receptor MT₂, a G protein–coupled receptor (GPCR). Karamitri et al. measured the spontaneous and melatonin-stimulated signaling of 40 different MT₂ variants. Computational analysis of these signaling profiles and assessment of genetic association data showed that those MT₂ variants with defective melatonin-stimulated G protein signaling and reduced spontaneous β-arrestin recruitment were associated with the greatest risk for T2D. These data may aid in the development of specific treatments for T2D depending on the patient’s MT₂ variant. Moreover, the experimental approach may be applied to assess the impact of other GPCR mutations on disease associations.