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Article

The human L-type calcium channel $Ca_v1.3$ regulates insulin release and polymorphisms in *CACNA1D* associate with type 2 diabetes

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Abstract

Aims/hypothesis Voltage-gated calcium channels of the L-type have been shown to be essential for rodent pancreatic beta cell function, but data about their presence and regulation

in humans are incomplete. We therefore sought to elucidate which L-type channel isoform is functionally important and its association with inherited diabetes-related phenotypes.

Methods Beta cells of human islets from cadaver donors were enriched using FACS to study the expression of the genes encoding voltage-gated calcium channel (Ca_v)1.2 and Ca_v1.3 by absolute quantitative PCR in whole human and rat islets, as well as in clonal cells. Single-cell exocytosis was monitored as increases in cell capacitance after treatment with small interfering (si)RNA against *CACNA1D* (which encodes Ca_v1.3). Three single nucleotide polymorphisms (SNPs) were genotyped in 8,987 non-diabetic and 2,830 type 2 diabetic individuals from Finland and Sweden and analysed for associations with type 2 diabetes and insulin phenotypes.

Results In FACS-enriched human beta cells, *CACNA1D* mRNA expression exceeded that of *CACNA1C* (which encodes Ca_v1.2) by approximately 60-fold and was decreased in islets from type 2 diabetes patients. The latter coincided with diminished secretion of insulin in vitro. *CACNA1D* siRNA reduced glucose-stimulated insulin release in INS-1 832/13 cells and exocytosis in human beta cells. Phenotype/genotype associations of three SNPs in the *CACNA1D* gene revealed an association between the C allele of the SNP rs312480 and reduced mRNA expression, as well as decreased insulin secretion in vivo, whereas both rs312486/G and rs9841978/G were associated with type 2 diabetes.

Conclusion/interpretation We conclude that the L-type calcium channel Ca_v1.3 is important in human glucose-induced insulin secretion, and common variants in *CACNA1D* might contribute to type 2 diabetes.

Keywords Beta cell • *CACNA1D* • Calcium • Ca_v1.3 channel • Diabetes • Exocytosis • Human • Insulin • Islets

Abbreviations

AU	Arbitrary unit
Ca_v	Voltage-gated calcium channel
GSIS	Glucose-induced insulin secretion
GWAS	genome-wide association studies

L-VGCC	L-type voltage-gated calcium channel
PPP	Prevalence, Prediction and Prevention of Diabetes study
qPCR	Quantitative PCR
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
VGCC	Voltage-gated calcium channel

Introduction

Insulin secretion requires the presence and activity of voltage-gated calcium channels [VGCCs; 1]. Various VGCC subclasses are described and the L-type class (L-VGCC) has been considered essential for insulin secretion [2]. However, which of the two main L-type isoforms ($Ca_v1.2$ and $Ca_v1.3$) is most important appears to be species dependent and to differ between rodents and humans [3]. Conclusive data showing gene expression in human beta cells has not previously been available.

In mice, global knockout of *Cacna1c* (which encodes $Ca_v1.2$) is lethal [4], whereas beta cell-specific disruption has demonstrated that $Ca_v1.2$ is critical for insulin release [5, 6], especially during the first phase [7]. $Ca_v1.3$, on the other hand, has been proposed to play a central role during murine postnatal beta cell generation and proliferation [8], but is also differentially regulated in a diabetogenic diet mouse model [9]. This isoform is characterised by a more negative activation threshold [10] and decreased calcium-channel blocker sensitivity [11] compared with $Ca_v1.2$, and has been suggested to be the main L-VGCC responsible for insulin release in rat beta cells [12, 13].

In humans, perturbed L-VGCC expression and function can cause disease, as exemplified by Timothy syndrome (caused by alterations in $Ca_v1.2$, encoded by *CACNA1C*) and congenital stationary night blindness (caused by dysfunctional $Ca_v1.4$, encoded by *CACNA1F* [14]). For human islets, mRNA expression of *CACNA1D* (encoding $Ca_v1.3$ protein) has been reported to exceed that of *CACNA1C* by tenfold [15]. Human genetic studies also point to *CACNA1D* being the most significant isoform for type 2 diabetes mellitus. A comparison between DNA from healthy individuals and those with type 2 diabetes revealed that, in the latter, *CACNA1D* carried an additional methionine-encoding triplet immediately downstream of the 5′

untranslated region [16, 17]. Although the functional consequences were not clarified, this finding supports the hypothesis of a causal link between genetic variations in the $Ca_v1.3$-encoding gene *CACNA1D* and risk for type 2 diabetes.

To evaluate this idea, we analysed data from genome-wide association studies (GWAS) for type 2 diabetes and quantitative traits [18, 19] in which we detected associations of single nucleotide polymorphisms (SNPs) in *CACNA1D* with type 2 diabetes. If and how these SNPs translate into changes in beta cell function have until now remained unanswered. Using genetics, molecular biology and electrophysiological approaches, we here present the first study determining the functional role of $Ca_v1.3$ for insulin secretion in the human beta cell and for human type 2 diabetes.

Methods

Real-time PCR RNA was extracted and reverse transcribed as previously described [20]. For absolute quantification, we produced standard curves using custom-designed DNA oligonucleotides (4 nM; idtDNA, Coralville, IA, USA). Transcript numbers are expressed as transcripts/ng reverse transcribed mRNA. For primer sequences, see electronic supplementary material (ESM) Table 1.

FACS of beta cells Beta cells from dispersed human islets were enriched using the Zn^{2+} dye Newport Green [21]. Apoptotic cells were accounted for (7-AAD staining) and islet cells were sorted using BD Biosciences FACSAria (BD Biosciences, San Jose, CA, USA). Beta cell enrichment was verified by immunohistochemistry and insulin-positive cells accounted for 90±5% of all cells in the beta cell fractions, comparable with previous observations [22].

Microarrays of human pancreatic islet mRNA RNA was isolated and prepared and the microarrays were performed and analysed as described in [23].

Only non-diabetic individuals were considered for correlations with *CACNA1D* expression. The genes that showed positive correlation (cut-off Pearson's $r \geq 0.8$) with *CACNA1D* expression (163 genes) were analysed using the DAVID database for functional annotation (david.abcc.ncifcrf.gov). The gene ontology category with the highest stringency for specific classification of gene product function (GOTERM_BP_5 in the gene ontology classification chart) was used and functions were sorted according to Fisher's exact *p* value (minimum gene count threshold of 5). All microarray data are presented as arbitrary $\log_2$ units (AUs).

Immunocytochemistry and microscopy Islets were treated as previously described [24] and incubated overnight at 4°C with anti-$Ca_v1.3$ (17 μg/ml, Cat. no. ACC-005, Lot no. AN-15, Alomone Labs, Jerusalem, Israel) and anti-insulin (1:200, Cat. no. 2263B65-1, Lot no. HS1761, EuroProxima, Arnhem, the Netherlands) or anti-glucagon (1:200, Cat. no. 2263B-GP310-1, Lot no. LN2051, EuroProxima) antibodies. Incubation with secondary antibodies anti-rabbit-DyLite488 and anti-guinea pig-Cy5 (1:200; Jackson ImmunoResearch, West Grove, PA, USA) for 4 h at RT and nuclei staining with Hoechst 33258 (1:1,000) was followed by visualisation in multitrack mode with a Zeiss 510 LSM confocal microscope with a C-Apochromat 40×/1.2 WDICIII lens (Carl Zeiss, Oberkochen, Germany).

Human study populations Non-diabetic participants from the Botnia Family Study (IVGTT, *n*=766) and the population-based Prevalence, Prediction and Prevention of Diabetes (PPP)-Botnia Study (OGTT, *n*=4,671) were used to investigate effects of genetic variants in *CACNA1D* on insulin secretion in vivo (Table 1) [25, 26]. First-phase insulin release was determined as previously described [25]. Insulin and glucose measurements were also performed as previously described [26]. Insulin readouts were converted (1 μU/ml = 6.945 pmol/l) and $\log_e$ transformed. The Malmö case–control population contained 2,830 patients with diabetes from the Malmö Diabetes Registry [27] and 3,550 non-diabetic controls from the Malmö Diet and Cancer study (Table 1) [28]. All cases were of Scandinavian origin, with an age at diabetes onset of older than 35 years, C-peptide levels of 0.3 nmol/l or higher and no GAD antibodies. Controls had fasting blood glucose less than 5.5 mmol/l, HbA_{1c} less than 6.0% (42 mmol/mol) and no known first-degree relatives with diabetes. The diagnosis of type 2 diabetes was based on WHO criteria [29]. All participants gave informed consent and the protocols were approved by the ethics committees of Jorbruksverket (Human islets and MCC), Central Hospital Vasa (Botnia) and Helsinki University Hospital (Botnia-PPP).

SNP selection and genotyping Genotyping was performed as previously described [23]. The SNPs analysed were not in linkage disequilibrium with each other and were chosen based on nominal associations with risk of type 2 diabetes in the Diabetes Genetics Initiative GWAS (rs9841978) [18] or the DIAGRAM+ meta-analysis for type 2 diabetes (rs312486 and rs9841978) [19], or based on their location and functional implication (rs312480 [16, 17, 30]; rs312486 as proxy for rs312481 [31]) (see ESM Table 2 for details). The genotyping success rate was higher than 97% (5% random replicates) and Hardy–Weinberg equilibrium was met in all studied populations.

In vitro insulin-release measurements, cell and islet culture Human pancreatic islets were supplied by the Nordic Network for Clinical Islet Transplantation (courtesy of O. Korsgren, Uppsala University, Uppsala, Sweden). Insulin-release measurements were conducted as previously described [32]. Culture of INS-1 832/13 cells and tests for glucose-induced insulin secretion (GSIS) were performed as previously described [20]. Diagnosis of type 2 diabetes was based on donor history records or as indicated otherwise (i.e. based on WHO criteria). HbA_{1c} was determined from patient blood samples taken while on life support.

Days in culture had no influence on *CACNA1D* expression in human islets. Although islet purity correlated significantly with *CACNA1D* expression ($p<0.01$), the mean purity between islets from control and diabetic participants was not significantly different (68±2% in controls vs 64±6% in diabetic participants).

Transfection with siRNA The small interfering (si)RNA oligonucleotides (rat siRNA ID s132089; human s2286) were supplied by Ambion (Austin, TX, USA). The negative control siRNA was non-targeting siRNA#1 (Cat. no. D-001810-01-05) from Dharmacon (Lafayette, LA, USA), as was the transfection reagent Dharmacon-1 (Cat. no. T-2001). Transfection was performed as previously described [20], but a concentration of 25 μM was used in the current study.

Electrophysiology Human islets were dispersed into single cells by incubation in Ca^{2+}-free buffer followed by trituration and overnight incubation in RPMI1640 medium containing 5 mmol/l glucose. They were then transfected with siRNA (see above) with additional co-transfection of a green fluorescent oligonucleotide (Cat. no. 2013; Invitrogen, Carlsbad, CA, USA) to identify successfully transfected cells. After 48 h, cells were used for electrophysiological capacity measurements as previously described [15].

Ca^{2+} imaging Imaging was performed using a Polychrome V monochromator (TILL Photonics, Graefeling, Germany) on a Nikon Eclipse Ti Microscope (Nikon, Tokyo, Japan). A ER-BOB-100 trigger, iXON3 camera and software iQ2 (Andor Technology, Belfast, UK) were used for control and detection. After 24 h culture in poly-L-lysine-coated glass-bottom dishes in indicated glucose concentrations, human islets were loaded with Fura-2 (2 μmol/l in KRB buffer containing 5 mmol/l glucose) for 35–40 min, left in the imaging system for 15 min while perfused with 37°C buffer prior to imaging. The exposure time was 50 ms at 2 s intervals. Islets were exposed to 5 mmol/l glucose for less than 5 min (time interval 1 [t_{i1}]), to 20 mmol/l glucose for 10 min (t_{i2}), 5 mmol/l glucose for 5 min (t_{i3}), 5 mmol/l glucose and

70 mmol/l KCl for 5 min (t_{i4}) and 5 mmol/l glucose for 5 min (t_{i5}). The AUC for the above time intervals was calculated as $AUC_{ti} = \Sigma[0.5 \times (t_n - t_{n-1}) \times (Ratio340/380_n + Ratio340/380_{n-1})]$.

Statistical analyses Data are presented as means±SEM. Quantitative traits were calculated using linear regression analysis corrected for age, sex and BMI. Type 2 diabetes risk was assessed using logistic regression adjusted for age, sex and BMI and is expressed as OR. p values were adjusted for the number of SNPs tested. All other data were statistically evaluated using an unpaired two-tailed Student's *t* test (unless otherwise indicated) after verification of equal variances and normal distribution of the data. For human islet quantitative (q)PCR and microarray data, an unpaired two-tailed *t* test assuming unequal variances was used with confirmation by Mann–Whitney *U* test. $p<0.05$ was considered significant.

Results

CACNA1C and CACNA1D expression in islets and beta cells Reports on L-VGCC gene expression are not conclusive and we therefore quantified expression of *Cacna1c* and *Cacna1d* in rat islets, INS-1 832/13 cells and *CACNA1C and –D* in human islets with qPCR. In whole rat islets, we found 998±197 *Cacna1c* mRNA transcripts (given as transcripts per ng reverse-transcribed RNA) and 9,727.9±2,629.8 *Cacna1d* transcripts (Fig. 1 a). Expression in INS-1 832/13 cells was similar to that observed in rat islets, with 1,580±337 transcripts for *Cacna1c* and 4,266±749 copies of *Cacna1d* (Fig. 1 b). We then enriched beta cells from three islet donors by FACS using dispersed human islet cells and the Zn^{2+} dye Newport Green DCF [21]. For comparison, transcripts were also quantified in undispersed human islets of the same individuals, and for *CACNA1C* they amounted to 7±2 (Fig. 1 c) compared with 19±1 in beta cells alone (Fig. 1 d). The number of *CACNA1D* mRNA molecules amounted to 550±30 in islets (Fig. 1 c) and 1,130±23 in human beta cells (Fig. 1 d), suggesting *CACNA1D* transcripts to be mainly localised to beta cells. Staining for $Ca_v1.3$ protein in whole islets revealed $Ca_v1.3$ immunoreactivity not only in insulin-positive (beta) cells (Fig. 1 i, k), but also in alpha cells (Fig. 1 j).

We then asked if *CACNA1D* expression was different in the islets of type 2 diabetes patients compared with controls. In microarrays *CACNA1D* mRNA was reduced in type 2 diabetes islets compared with controls (7.27±0.16 AU in 10 cases vs 7.69±0.55 AU in 66 controls; $p=0.03$; *t* test; Fig. 1 e). *CACNA1C* expression was not significantly changed but

directionality was the same (6.22±1.39 AU in cases vs 6.47±0.04 in controls; Fig. 1 h). Interestingly, the expression of $Ca_v1.2$ and *CACNA1D* showed strong covariation (Pearson's r=0.9; $p=2.5\times10^{-28}$; not shown). The downregulation of *CACNA1D* in type 2 diabetes islets was confirmed with absolute qPCR (1,235±64 *CACNA1D* transcripts in 63 controls vs 754±93 in seven cases; $p=2\times10^{-4}$, *t* test; Fig. 1 f). Similar results were obtained using HbA_{1c} levels to define diabetic status, with *CACNA1D* expression being 50% lower in individuals with HbA_{1c} of 6.5% (=48 mmol/mol) or higher (626±133 vs 1,250±88 transcripts when HbA_{1c}<6.5% [48 mmol/mol]; p=0.008, *t* test; Fig. 1 g). We found no significant change in *INS* expression in type 2 diabetes islets as compared with controls ($2^{-\Delta C_t}$ INS relative to beta-actin $3.4\times10^{-5}\pm1.5\times10^{-5}$ in cases vs $4.6\times10^{-5}\pm6.7\times10^{-6}$ in controls, p=0.25). This suggests that the reduced *CACNA1D* expression in islets from hyperglycaemic/type 2 diabetes donors cannot be attributed to reduced islet mass or reduced beta cell fraction within the islets, in agreement with previous thorough investigations of the same islet donor cohort ([33, 34]).

Effect of genetic variants in CACNA1D on insulin secretion and type 2 diabetes The expression of the related L-VGCC gene *CACNA1C* has been shown to be genetically determined by SNPs [35], giving a functional explanation for associations of *CACNA1C* genotypes with neurological disorders [36, 37]. Similarly, to determine if the changes in expression of the $Ca_v1.3$ Ca^{2+} channel gene *CACNA1D* that we observed in human pancreatic islets also are influenced by genetic variations, we studied SNPs in *CACNA1D* and their associations with gene expression as well as with insulin release and type 2 diabetes.

We used literature searches and previously published GWAS studies to select the best candidate SNPs in *CACNA1D* ±50 kb (see Methods and ESM Table 2 for details).

The first marker analysed was rs312480 and the C allele associated with decreased 30 min insulin concentration (IVGTT; β=–0.103; p=0.036, Table 2 and Fig. 2 a). The same directionality (β) was observed for 30 min insulin during the Botnia-OGTT Study (Table 2), although values were not statistically significant. This suggests that the additional mechanisms of insulin release that are in operation when administering glucose via the oral route (e.g. the incretin effect) may obscure the direct effects of rs312480 on 30 min insulin values observed during IVGTT. Although we found no significant effect on fasting insulin in the smaller Botnia-OGTT Study, we detected decreased fasting insulin values in the OGTT of the larger PPP-Botnia Study (β=–0.047; p=0.048) due to the larger sample size and therefore increased power.

In line with these in vivo findings, rs312480/C showed reduced in vitro insulin release in batch incubations at high glucose (16.7 mmol/l; CC 1.4±0.1 vs CT 2.2±0.3 ng/islet×h, p=0.04; T/T n.a.; Fig. 2 b). Microarray data from islets of the same individuals revealed a concomitant reduction in *CACNA1D* mRNA in the carriers of CC genotypes (CC 7.6±0.1 vs CT 8.0±0.2 AU, p=0.03; Fig. 2 c).

Neither the variant rs9841978 nor rs312486 showed a significant association with insulin secretion (ESM Table 3) or with changes in gene expression. However, we next wanted to examine if any of these SNPs in *CACNA1D* that were previously suggested to be associated with disease would also have an effect on risk of type 2 diabetes. In the Malmö case–control population, including 6,570 individuals (2,830 type 2 diabetes cases and 3,740 controls), rs9841978/G (OR 1.16, 95% CI 1.06, 1.27; p=0.003) and rs312486/G (OR 1.17, 95% CI 1.06, 1.3; $p=1.9\times10^{-4}$) were indeed associated with an increased risk of type 2 diabetes. The genotype frequencies for control (type 2 diabetes) individuals for rs312486 were 61.9% (66.9%) for G/G, 33.7% (29.4%) for G/C and 4.4% (3.7%) for C/C. For rs9841978, they amounted to 48.1% (53.4%) for G/G, 41.9% (38.2%) for G/A and 10.1% (8.4%) for A/A genotype carriers. We verified our findings by meta-analysis of DIAGRAM+ (see ESM Table 2) and our own results. For rs312486/G, we found an OR of 1.11 (95% CI 1.06, 1.16 $p=1.1\times10^{-5}$) and for rs9841978/G the OR was 1.08 (95% CI 1.04, 1.12; $p=1.1\times10^{-4}$). The reason why the markers rs9841978 and rs312486 exhibited no significant association with insulin secretion or gene expression, but an increased risk of type 2 diabetes, remains unclear, but may be because the risk of type 2 diabetes is not solely related to impaired beta cell function or because of confounding effects on survival.

Glucose effects Given that *CACNA1D* transcript numbers were decreased in islets of individuals with type 2 diabetes, we next investigated the possible direct effects of glucose on *CACNA1D* expression in human islets. After 24 h incubation, insulin concentrations in the medium were 5.5-fold higher at 20 mmol/l glucose as compared with 5 mmol/l glucose, but in the presence of the L-type calcium-channel blocker isradipine the islets failed to release insulin (p=0.02, ESM Fig. 1), as also reported elsewhere [15].

After 24 h islets were rested at 5 mmol/l glucose for 1 h then stimulated with 20 mmol/l glucose. Islets that had been cultured for 24 h in 20 mmol/l glucose now released significantly more insulin than islets cultured at basal (5 mmol/l) glucose (4.2±0.4-fold vs 2.6±0.4-fold, p<0.01; Fig. 3 a). The presence of isradipine in the 24 h incubation medium

completely abolished GSIS in subsequent batch incubations. We next asked what effect this L-type channel inhibition had on *CACNA1D* mRNA expression. Absolute quantification revealed that high glucose increased *CACNA1D* transcript numbers approximately twofold, whereas expression levels remained unaffected in the presence of isradipine (Fig. 3 b). Increased *CACNA1D* expression coincided with elevated basal $[Ca^{2+}]_i$ levels (2.14±0.04 AUCi for islets cultured for 24 h at 20 mmol/l vs 1.68±0.04 when tested at 5 mmol/l glucose; $p=8\times10^{-4}$; Figs. 3c, d), an exaggerated response to 20 mmol/l glucose (6.09±0.09 for 20 mmol/l glucose vs 4.5±0.05 for 5 mmol/l; $p=1\times10^{-5}$) and higher levels during (6.51±0.39 vs 5.53±0.16; $p=0.046$) and after 70 mmol/l KCl stimulation (1.84±0.05 vs 1.44±0.05; $p=2\times10^{-3}$). Typical $[Ca^{2+}]_i$ oscillations during high-glucose stimulation were absent in the islets that had been incubated at 20 mmol/l glucose for 24 h (Fig. 3 d). The elevated expression of *CACNA1D* by long-term 24 h glucose treatment is thus functionally important and clearly affects $[Ca^{2+}]_i$ homeostasis.

As we detected decreased *CACNA1D* expression in the islets of individuals with type 2 diabetes, we wanted to examine whether the presence of *CACNA1D* transcripts was a prerequisite for appropriate glucose-induced insulin release. We used siRNAs against human *CACNA1D* (si*CACNA1D*), rat *Cacna1d* (si*Cacna1d*) and the rat beta cell line INS-1 832/13, a model for the study of GSIS [38]. The two siRNAs tested (designed for human and rat *CACNA1D*, but both targeting rat *Cacna1d*) were comparably efficient and after 48 h *Cacna1d* expression in INS-1 832/13 cells was decreased by 72±6% (Fig. 3 e, shown only for siRNA against rat *Cacna1d*; $p=0.02$) compared with the non-targeting siRNA. $Ca_v1.3$ protein was also decreased (Fig. 3 i). We then tested for effects on GSIS. While basal release was not affected (Fig. 3 f; 1.5±0.1 ng insulin/mg protein×h in si*Cacna1d*-treated vs 1.8±0.3 ng insulin/mg protein×h in control cells), the response to high glucose was significantly reduced in cells treated with si*Cacna1d* (Fig. 3 f; 3.8±0.4 ng/mg protein×h in si*Cacna1d* vs 6.7±0.7 ng/mg protein×h in control; $p=0.0026$). Human beta cells responded in a similar manner. After 48 h incubation with si*CACNA1D*, depolarisation-evoked single-cell exocytosis was significantly reduced when compared with control cells (Fig. 3 g, h; 41±4 fF in si*CACNA1D* vs 68±8 fF in control cells; $p=0.009$). The difference was significant for each of the ten depolarisations (not shown). Exocytosis was reduced by about 40%, comparable with the reduction of GSIS in the rat beta cell line.

CACNA1D and the exocytotic microdomain Our data indicate that the appropriate expression of *CACNA1D* and function of $Ca_v1.3$ are important determinants of GSIS. To further examine the role of $Ca_v1.3$ in the exocytotic process, we extended our microarray studies by exploring the genes that positively correlate with *CACNA1D* expression (cut-off Pearson's $r \geq 0.8$, 163 genes). We found that genes coexpressed with *CACNA1D* were significantly enriched for particular cellular functions (Table 3) and the top-ranked category was exocytosis ($p=9\times10^{-4}$). This is in agreement with the functional results and underlines the relevance of these findings.

Discussion

The L-type calcium channels of the beta cell have been extensively studied, but mostly in rodents. It therefore remains unclear which isoform is expressed and operational in the human beta cell. In this study, we set out to determine this, as well as possible associations between genetic variations and phenotypes related to type 2 diabetes. We demonstrate that $Ca_v1.3$ dominates over the related $Ca_v1.2$ channel on the mRNA level, and that $Ca_v1.3$ mRNA and protein are mainly localised to human beta cells. Second, we have shown that a SNP (rs312480) in *CACNA1D*, the gene encoding $Ca_v1.3$, can influence gene expression of the channel and affect insulin release, and that other SNPs (rs312486 and rs9841978) associate with type 2 diabetes. Furthermore, our data illustrate that the expression of *CACNA1D* mRNA is reduced in islets of patients with type 2 diabetes (Fig. 1) and that pharmacological or RNA interference-mediated inhibition of *CACNA1D* expression and/or $Ca_v1.3$ operation decreases GSIS and, on a single-cell level, exocytosis (Fig. 3).

Animal studies designed to investigate which L-type channel isoform is expressed and functional in beta cells have revealed that insulin release in the mouse mainly depends on $Ca_v1.2$ [6, 7, 39, 40], with a diminished first phase after ablation of the channel [7]. However, a compensatory upregulation of $Ca_v1.2$ upon *Cacna1d* knockout, as detected by another group [8], could not be ruled out [39]. Studies in rats claimed $Ca_v1.3$ to be the major L-VGCC involved [12], which is similar to what is proposed for humans [15], although neither study quantified protein levels. We detected $Ca_v1.3$ transcripts and protein not only in whole islets, but also found both to be enriched in beta cells, although some protein was also detected in alpha cells. We also identified a common variant in *CACNA1D* (SNP rs312480) to be associated with reduced fasting insulin and putatively also with 30 min insulin levels, indicating that this isoform might not strictly participate in phasic but also in basal insulin secretion. In fact, $Ca_v1.3$, with its lower activation threshold compared with $Ca_v1.2$ [10], has

been suggested to be involved in pacemaking in the heart [41] and in the neuroendocrine chromaffin cell [42]. Considering that the neuroendocrine beta cell also fires action potentials in the physiological resting (or preprandial) condition [43], $Ca_v1.3$, with its low activation threshold, might be a good candidate for these events. In the glutamate-releasing inner-ear hair cell, $Ca_v1.3$ has been shown to be responsible for the generation of spontaneous action potentials, important for the maturation of synaptic connections within the developing cochlea [44]. In a remarkable analogy, the postnatal maturation of mouse beta cells has also been suggested to be dependent on the presence of $Ca_v1.3$ [8], although spontaneous spiking during this process has never been investigated. Reflecting on our finding that a polymorphism in the gene encoding for $Ca_v1.3$ leads to decreased expression and reduced insulin release, our present data suggest that this is not related to a decrease in islet mass or beta cell proportion. The possibility that the genotype is associated with the capacity for postnatal human beta cell expansion, for instance in insulin resistance, requires live determination and longitudinal studies of beta cell mass, techniques that remain to be developed for application in humans [45]. Nevertheless, we here demonstrate that in beta cells of adult humans, $Ca_v1.3$ is important for insulin exocytosis as knockdown of its expression also reduced insulin vesicle fusion events (Fig. 3 f, g). This is in accordance with findings from Braun and colleagues [15], where the L-type blocker isradipine abolished glucose-induced insulin release in human islet batch incubations and reduced exocytosis in patch experiments, although the latter was even more suppressed by P/Q-type channel blockers. Nevertheless, isradipine completely inhibited glucose-induced action potentials, suggesting that a single depolarisation in voltage-clamp mode strongly underestimates the pharmacological effect of L-type channel blockage on beta cell function.

We also found that $Ca_v1.3$ knockdown reduced insulin release in INS-1 832/13 cells. A previous study suggested that $Ca_v1.2$ knockdown diminished GSIS [46] but as we did not test siRNA against $Ca_v1.2$, it may well be that both channels are crucial for appropriate insulin release in these cells.

Our study also reveals that human *CACNA1D* expression responds to a change in glucose levels, a phenomenon that has previously been observed in rats [13]. No data on the effects of glucose on *Cacna1d* expression in mice are available, but a several-week-long diabetogenic high-fat diet has been shown to decrease rather than increase *Cacna1d* expression [9]. However, we found increased levels after a high-glucose episode of just 24 h, whereas after

48 h Iwashima and colleagues detected a decline (in rats) [13]. Although another species and time frame, this indicates a putative time- and activity-dependent regulation of *CACNA1D* expression. Indeed, for the related $Ca_v1.2$ channel, a mechanism has been put forward whereby calpain Ca^{2+} dependently cleaves an (among L-type VGCC well conserved [47]) autoinhibitory C-terminal domain of the channel. The cleaved C-terminus disinhibits the channel and acts as a transcription factor that controls expression of the $Ca_v1.2$ encoding gene *CACNA1C* [48]. Interestingly, when we pharmacologically inhibited Ca^{2+} influx through L-type channels and consequentially also insulin release by isradipine (Fig. 2 a), *CACNA1D* expression was no longer augmented (Fig. 2 b). This suggests suppression of the activity-dependent positive-feedback loop that otherwise would drive the rise in channel expression analogous to that proposed for *Cacna1c* ($Ca_v1.2$). We have indeed observed that calpain inhibitor-1 can reduce the level of *CACNA1D* mRNA in the high-glucose condition back to isradipine levels (not shown). The observed glucose-induced activity-dependent rise in *CACNA1D* expression correlated with increased basal Ca^{2+} levels, as well as higher levels under stimulation (Fig. 3 c, d). Human islets cultured under high-glucose conditions for 48 h have previously been described to be desensitised due to increased basal Ca^{2+} levels and diminished slow $[Ca^{2+}]_i$ oscillations, and thus to respond poorly to glucose stimulation [49]. Interestingly, after 24 h we also found diminished Ca^{2+} oscillations but hypersensitisation rather than desensitisation (Fig. 3 d). It has been suggested that glucose causes hypersensitisation first, followed later by desensitisation and Ca^{2+}-induced apoptosis [glucotoxicity; 50] and the two findings may thus be in line with each other.

It is tempting to speculate that pharmacological inhibition of excessive Ca^{2+} influx in prediabetic islets, in order to prevent Ca^{2+} induced hypersensitisation, may be an option for early-stage intervention that might at least delay the onset of type 2 diabetes.

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Contribution statement

TMR conducted research and wrote the manuscript. SA conducted research and contributed to revision of the manuscript. EA, TT and BI conducted research and reviewed the manuscript. VL and ER contributed to the concept and design of the study, and reviewed and edited the manuscript. All authors approved the final version of the manuscript.

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

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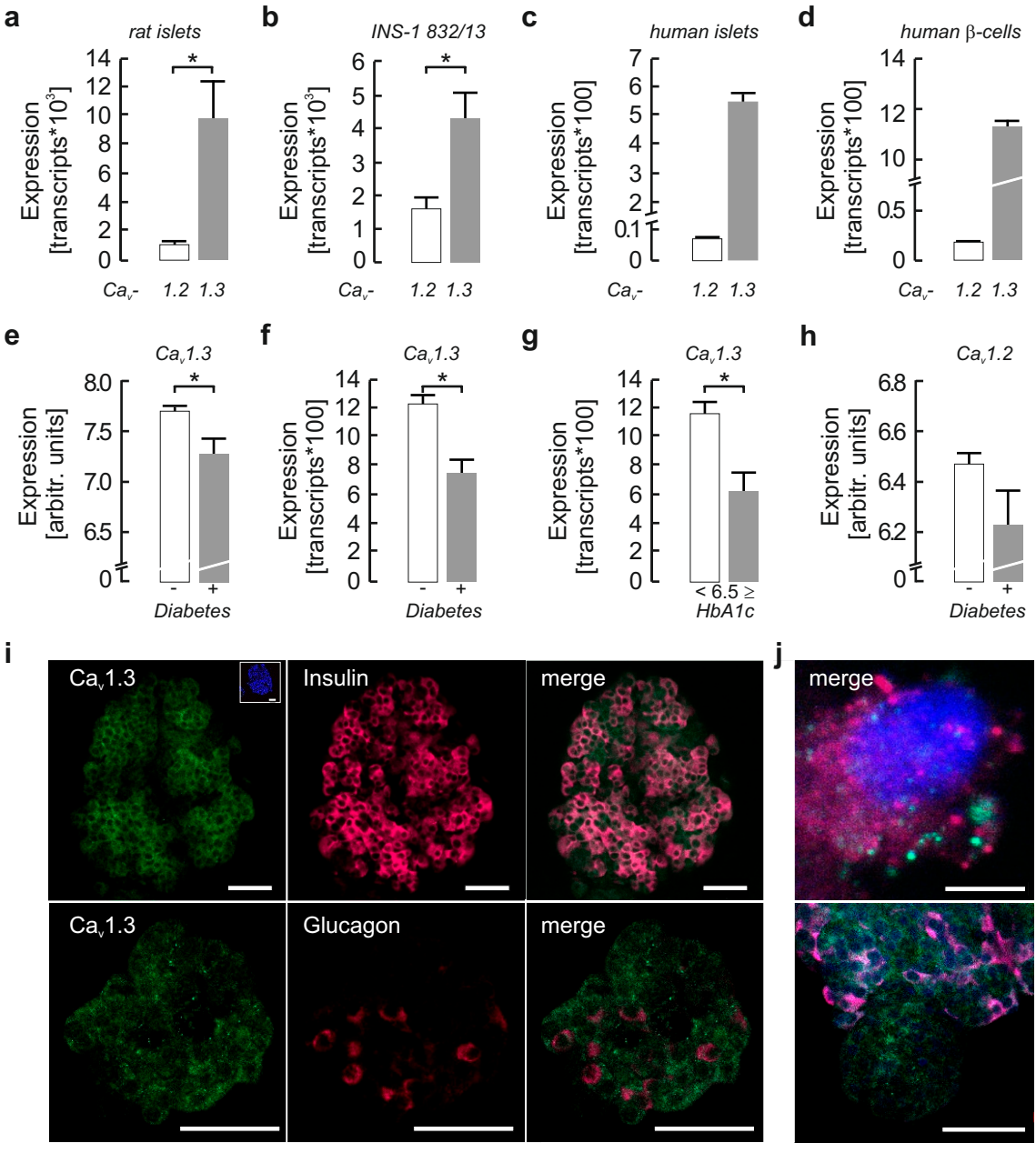
Figures and tables

Fig. 1 Expression of Cacna1c/*CACNA1C* (encoding rat/human $Ca_v1.2$) and Cacna1d/*CACNA1D* (rat/human $Ca_v1.3$). (**a**, **b**) mRNA expression of *Cacna1d* (grey bar) exceeds that of *Cacna1c* (white bar) in rat islets (**a**) as well as in INS-1 832/13 cells (**b**). The same relationship, but with greater difference, is found in human islets (**c**) and in human beta cells (**d**), where *CACNA1D* (grey bar) dominates over *CACNA1C* (white bar). (**e**, **f**) Microarray studies revealed that expression of *CACNA1D* (**e**) is reduced in islets of individuals with type 2 diabetes (grey bars) compared with controls (white bars), confirmed by qPCR (**f**). (**g**) Expression of *CACNA1D* is also reduced in islets from human organ donors with high HbA_{1c} (≥6.5% [48 mmol/mol]; n=6) as compared with those with low HbA_{1c} (<6.5% [48 mmol/mol]; n=42). (**h**) Expression of *CACNA1C* is not significantly changed but directionality is the same as for *CACNA1D*. (**i**,**j**) $Ca_v1.3$ immunoreactivity (green) is detected in insulin-positive cells (red, **i**) and in glucagon-positive cells of control islets (red, **j**). Note that only a few glucagon-positive cells are detected. (**i**) Inset: control with omitted primary antibody and final Hoechst 33258 staining. Scale bars 50 μm. (**k**) A single cell showing distinct staining for insulin (red) and $Ca_v1.3$ (green). Scale bar 5 μm. (**l**) Some small islets are $Ca_v1.3$ positive but lack insulin protein. Scale bar 50 μm. All data are means±SEM. *p<0.05. AU, arbitrary units. Expr., expression.

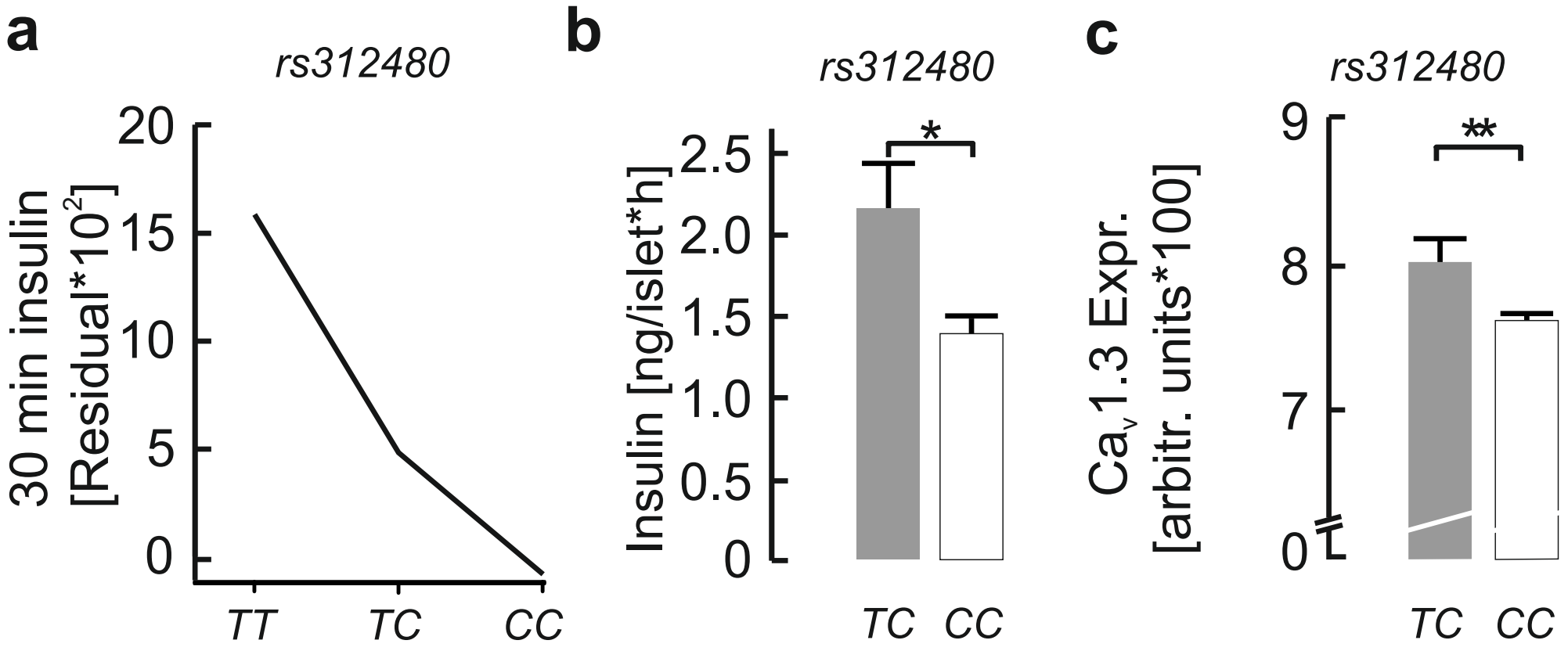
Fig. 2 (**a**) rs312480 has effects on in vivo and in vitro insulin levels and on mRNA expression. Thirty minute insulin levels (standardised residual corrected for age, sex and BMI shown) are reduced in rs312480/C allele carriers of the Botnia IVGTT Study (**a**) as well as in human islet batch incubations (**b**). (**c**) *CACNA1D* expression is decreased in the same individuals as in (**b**). All data are means±SEM. *p<0.05. AU, arbitrary units. Expr., expression.

Fig. 3 *CACNA1D* expression changes upon incubation with 20 mmol/l glucose (Glc.). Incubation of control human islets in medium with 20 mmol/l glucose for 24 h resulted in an increased fold insulin release (**a**, light grey bars) in a batch incubation and increased *CACNA1D* expression (**b**, light grey bars), compared with the 5 mmol/l glucose condition (white bars), unless the L-type Ca^{2+} channel inhibitor isradipine (Isr.) had been present for 24

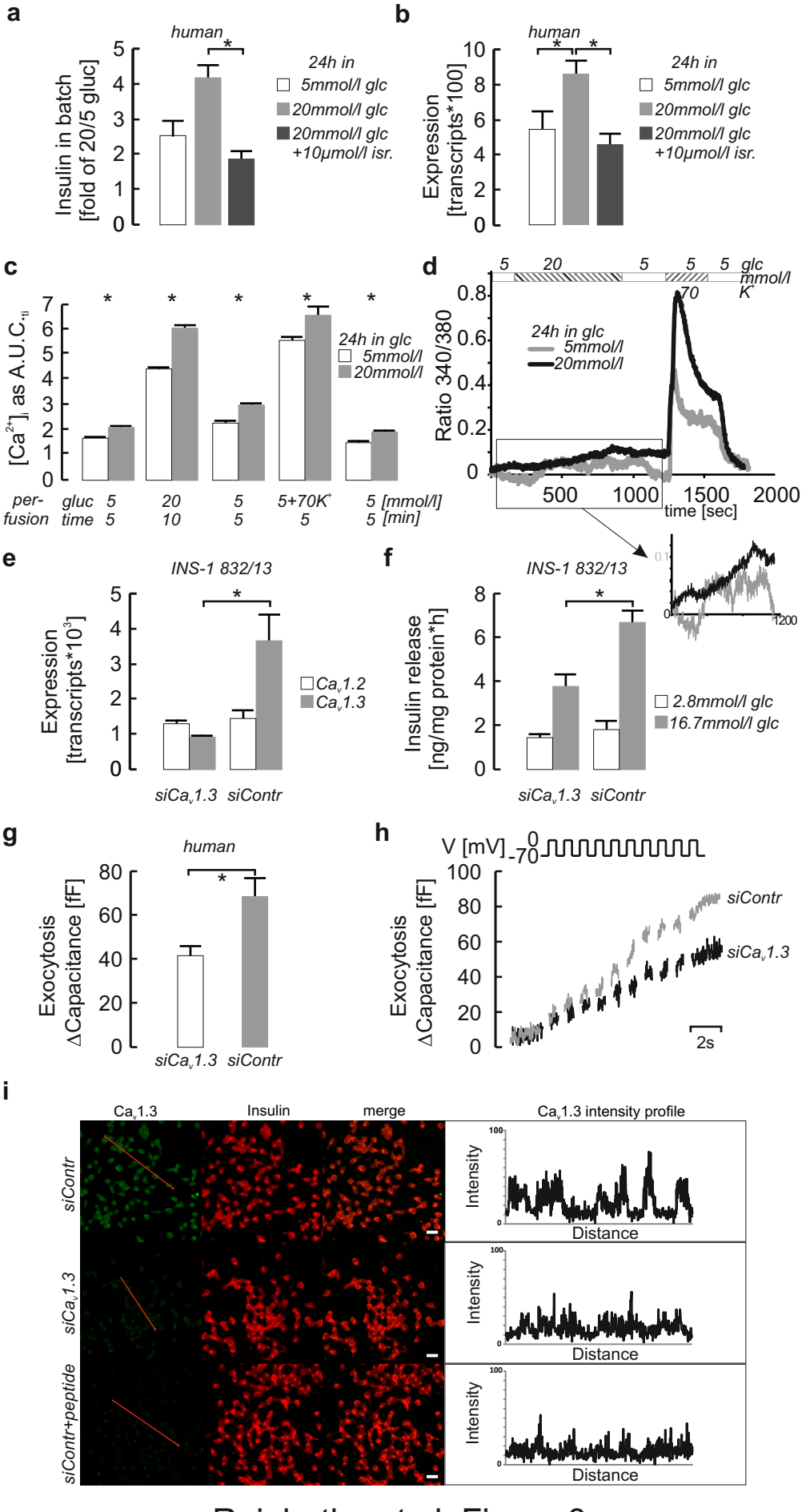
h (**a**, **b**; dark grey bars). No isradipine was added during the batch incubation. In parallel, intracellular Ca^{2+} levels were increased after 24 h in 20 mmol/l glucose (**c**, grey bars, given as the sum of the AUC [AUC_{ti}] in the given time intervals [5 or 10 min]) compared with 24 h in 5 mmol/l glucose (white bars). The example shown in (**d**) with inset depicts the absence of $[Ca^{2+}]_i$ oscillations during high-glucose stimulation in the islets incubated for 24 h at 20 mmol/l glucose (black traces) compared with 24 h in 5 mmol/l glucose (grey traces). (**e**) siRNA against *Cacna1d* reduced *Cacna1d* mRNA expression (grey bars) but not *Cacna1c* expression (white bars). (**f**) Glucose-induced insulin release (16.7 mmol/l) was reduced in siCa_v1.3-treated INS-1 832/13 cells (grey bars), whereas basal release was unaffected (2.8 mmol/l; white bars). (**g**) In analogy, in human beta cells, exocytosis was reduced after siCa_v1.3 treatment (white bar) compared with siContr (grey bar). A typical example is shown in (**h**), with the stimulation protocol being illustrated. (**i**) Ca_v1.3 knockdown (siCa_v1.3) in INS-1 832/13 cells also reduced Ca_v1.3 protein (Ca_v1.3) without an effect on insulin protein expression (Insulin) when compared with controls (siContr). Absence of Ca_v1.3 immunoreactivity upon pre-incubation with Ca_v1.3 peptide (**i**, siContr+peptide) verifies the specificity of the antibody. The intensity profiles depict the intensity of the red lines drawn in the corresponding left panels (Ca_v1.3). All data are means±SEM. *p<0.05 (Student's t test); siContr, control siRNA.



Reinbothe et al. Figure 1



Reinbothe et al. Figure 2



Reinbothe et al. Figure 3

Table 1 Clinical characteristics of the study participants.

Characteristic	Botnia population	PPP-Botnia population	Malmö case–control population	
	Controls	Controls	Controls	Cases
n (male/female)	766 (358/408)	4,671 (2,173/2,498)	3,550 (1,340/2,210)	2,830 (1,667/1,163)
Age (years)	49±12	48±15	57±6	63±11
BMI (kg/m^2)	28±4	26±4	25±4	28±9

Data are means±STDEV

Table 2 Effects of *CACNA1D* SNP rs312480 on insulin secretion during IVGTT and OGTT analysed by linear regression.

Study	rs312480 (C allele)			
	β	SE	p	Corr p[a]
Botnia Family Study				
IVGTT				
Fasting insulin (pmol/l)[b]	–0.590	0.146	0.027	0.081
30 min insulin (pmol/l)[b]	–0.715	0.160	0.012	0.036
60 min insulin (pmol/l)[b]	–0.583	0.194	0.038	0.114
First-phase insulin release[b]	0.479	0.201	0.089	0.267
OGTT				
Fasting insulin (pmol/l)[b]	–0.486	0.118	0.071	0.213
30 min insulin (pmol/l)[b]	–0.153	0.160	0.578	1.734
60 min insulin (pmol/l)[b]	–0.472	0.160	0.083	0.249
120 min insulin (pmol/l)[b]	–0.257	0.194	0.342	1.026
PPP-Botnia Study				
Fasting glucose (mmol/l)	0.005	0.018	0.748	2.244
30 min glucose (mmol/l)	0.013	0.051	0.378	1.134
120 min glucose (mmol/l)	–0.010	0.052	0.524	1.572

Fasting insulin (pmol/l)[b]	–0.250	0.056	0.001	0.003
30 min insulin (pmol/l)[b]	0.090	0.056	0.405	1.215
120 min insulin (pmol/l)[b]	0.042	0.083	0.686	2.058

β indicates directionality

[a]*p* value corrected for the number of SNPs analysed

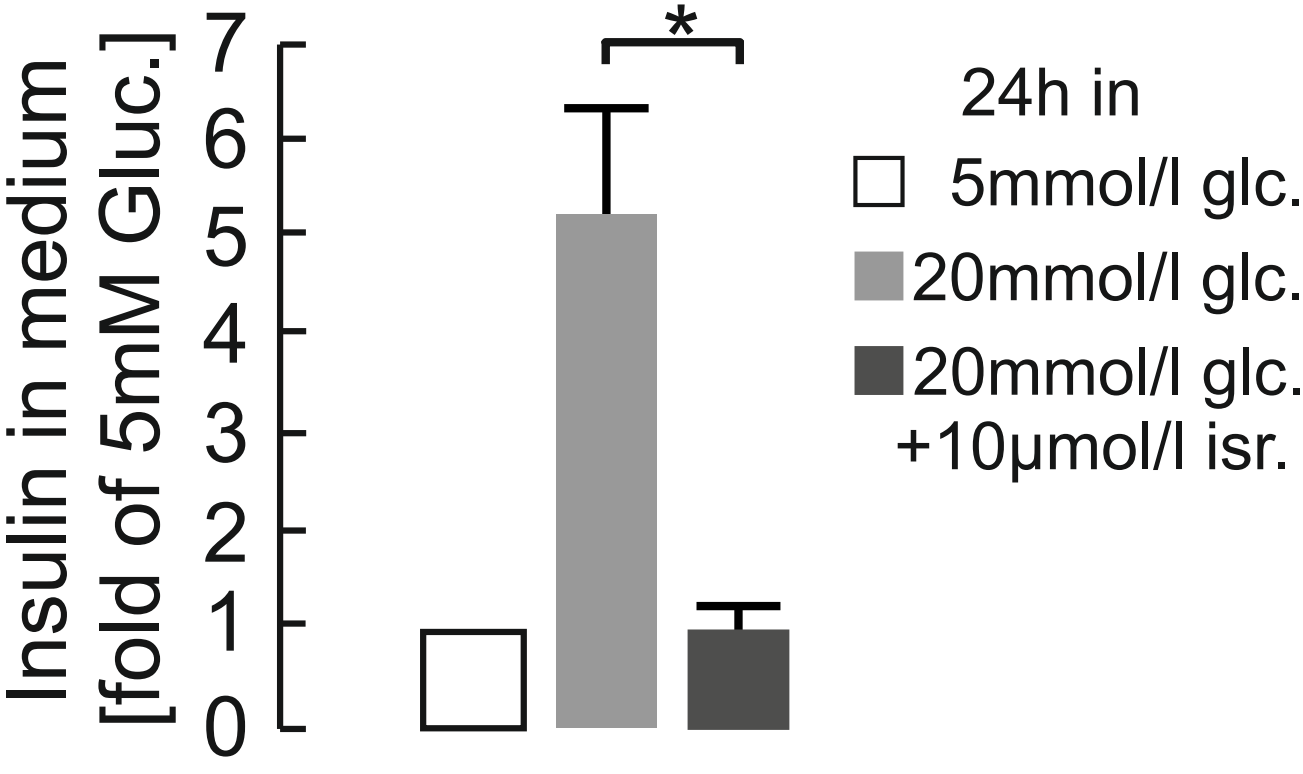
[b]Log_e transformed

Table 3 Gene ontology analysis of the genes positively correlating with *CACNA1D* expression in control human islets (Pearson's $r \geq 0.8$; 163 genes). The three highest scoring categories of gene product functions are depicted

Term	Fold enrichment	*p*
Exocytosis	5.3	9×10^{-4}
Axonogenesis	5.3	2×10^{-5}
Cell morphogenesis involved in neuron differentiation	4.9	4×10^{-5}

Fisher's exact corrected *p* values are shown

ESM Figure 1



Human islets were incubated for 24h in RPMI medium supplemented with the glucose concentrations indicated and insulin concentration in the medium was analysed using RIA. Concentrations are given relative to the 5mM glucose condition (white bar). glc=glucose; isr.=isradipine.

ESM Table 1. Primers used for quantitative realtime PCR. SG denotes the realtime PCR method using SYBR Green and TM denotes the Taqman® gene expression assays (Cat. no. of Life Technologies, Carlsbad, CA, USA is given). All were designed across one or two exon-exon boundaries to ensure amplification only of cDNA and not gDNA. Insulin primers were those described previously[*]. Similar efficiencies (>0.95) and sensitivities were tested for.

mRNA	*Species*	*Forward primer*	*Reverse primer*	*System*
Cacna1c	Rat	Rn00709287_m1		TM
Cacna1d	Rat	Rn01453378_m1		TM
CACNA1C	Human	CTCCTTCAGGAA CCATATTCTGTT	GCATTGCCTAG GATCTTCAGAG	SG
CACNA1D	Human	AGGGTAACTCGT CCAACAGC	TGTCAAATGGTT TCCATTCC	SG
INS	Human	GCAGCCTTTGTG AACCAACA	TTCCCCGCACAC TAGGTAGAGA	SG
ACTB/Actb	Human/ Rat	TGACCCAGATCA TGTTTGAGA	CAGGTCCAGAC GCAGGAT	SG

References: [*]Kirkpatrick et al.; PLoS One 5(6), 2010

ESM Table 2: Rationale for selection of SNPs and relevance to previous studies and phenotypes. The 3 SNPs chosen were not in LD with each other. The region analysed was +/-50kbp from *CACNA1D*. Rs312480 is located in the 5'UTR of exon 1, close to a reported trinucleotide repeat occurring in type 2 diabetes patients[1,2]. The 5'UTR is a major site for post-transcriptional regulation and has a strong disease relevance[3]. Rs312486 is positioned in intron 3 and is used as a proxy for rs312481 as assay design for rs312481 failed (LD=1). Rs312481 associates with L-type Ca^{2+} channel blocker sensitivity in hypertensive subjects[5], indicating a genetic effect on $Ca_v1.3$ channel function. Both rs312486 and rs312481 exhibit nominal type 2 diabetes association in the DIAGRAM+ study[4]. Marker rs9841978 is found in intron 8, associates with type 2 diabetes and is in strong LD with other SNPs that associate with type 2 diabetes[4,6]. Interestingly, the adjacent exon 8 is alternatively spliced (8a/8b). Some of the GWAS data cited is no longer available as supplementary material online, but is available on request from the publishers. DHP, L-type Ca^{2+} channel blockers of the Dihydropyridine class. T2D, Type 2 diabetes.

SNP ID	*Study (or name)*	*Associated phenotype*	*p-value*	*Reference*
rs312480	Functional+Genetic	ATG repeat in T2D in exon1; 5'UTR role in disease	-	[1,2,3]
rs312486	DIAGRAM+	Type 2 diabetes	9x10-4	[4]
	Kamide et al.	Reduced DHP sensitivity in hypertensive subjects	3x10-2	[5]
rs9841978	DIAGRAM+	Type 2 diabetes	6x10-3	[4]
	DGI	Type 2 diabetes	1x10-2	[6]

References: [1]Yamada et al., Genomics 27(2), 1995; [2]Yamada et al., Diabetes Metab Res Rev. 17(3), 2001; [3]Pickering and Willis, Semin Cell Dev Biol. 16(1), 2005; [4]Voight et al., Nat Genet. 42(2), 2010; [5]Kamide et al., Cir J. 73(4), 2009; [6]Saxena et al., Science 316(5829), 2007.

ESM Table 3: Effects of *CACNA1D* SNP rs312486 and rs9841978 on insulin secretion during IVGTT and OGTT. β indicates directionality. SE, Standard error. Corr p, p-value corrected for number of SNPs analysed.

	rs312486 (C-allele)				rs9841978 (A-allele)			
Botnia	β	SE	p	corr p	β	SE	p	corr p
IVGTT								
fasting insulin (μU/l)*	-0.653	0.153	0.085	0.255	-0.431	0.146	0.27	0.810
30 min insulin (μU/l)*	0.361	0.181	0.369	1.107	-0.403	0.167	0.324	0.972
60 min insulin (μU/l)*	-0.097	0.201	0.799	2.397	-0.069	0.188	0.859	2.577
FPIR*	-0.097	0.229	0.825	2.475	0.174	0.181	0.7	2.100
OGTT	β	SE	p		β	SE	p	
fasting insulin (μU/l)*	0.465	0.139	0.231	0.693	0.271	0.132	0.51	1.530
30 min insulin (μU/l)*	-0.021	0.194	0.959	2.877	-0.257	0.174	0.552	1.656
60 min insulin (μU/l)*	-0.042	0.188	0.917	2.751	-0.069	0.174	0.874	2.622
120 min insulin (μU/l)*	0.076	0.243	0.86	2.580	0.583	0.222	0.171	0.513
PPP-Botnia	β	SE	p		β	SE	p	
fasting glucose (mmol/l)	0.000	0.015	0.976	2.928	-0.014	0.012	0.333	0.999
30 min glucose (mmol/l)	-0.004	0.042	0.783	2.349	-0.017	0.035	0.240	0.720
120 min glucose (mmol/l)	0.007	0.043	0.605	1.815	-0.002	0.035	0.892	2.676
fasting insulin (μU/l)*	-0.035	0.042	0.728	2.184	0.028	0.035	0.764	2.292
30 min insulin (μU/l)*	-0.188	0.049	0.057	0.171	0.063	0.042	0.535	1.605
120 min insulin (μU/l)*	-0.069	0.069	0.491	1.473	0.063	0.265	0.531	1.593

*$\log_e$ transformed