

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.

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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 the more 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 *CACNAID* (encoding $Ca_v1.3$ protein) has been reported to exceed that of *CACNA1C* by tenfold [15]. Human genetic studies also point to *CACNAID* 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, *CACNAID* 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 *CACNAID* 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 *CACNAID* 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 $90 \pm 5\%$ 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 *CACNAID* expression. The genes that showed positive correlation (cut-off Pearson's $r \geq 0.8$) with *CACNAID* 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 $\mu\text{g/ml}$, 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 $40\times/1.2$ 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 ($1 \mu\text{U/ml}=6.945 \text{ pmol/l}$) 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 ($68\pm 2\%$ in controls vs $64\pm 6\%$ 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].

Ca²⁺ 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 $\mu\text{mol/l}$ 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_1]), to 20 mmol/l glucose for 10 min (t_2), 5 mmol/l glucose for 5 min

Table 1 Clinical characteristics of the study participants

Characteristic	Botnia population Controls	PPP-Botnia population Controls	Malmö case–control population	
			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 \pm 12	48 \pm 15	57 \pm 6	63 \pm 11
BMI (kg/m^2)	28 \pm 4	26 \pm 4	25 \pm 4	28 \pm 9

Data are means \pm SD

(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 $AUC_{ti} = S [0.5 \times (t_n - t_{n-1}) \times (\text{Ratio}_{340/380_n} + \text{Ratio}_{340/380_{n-1}})]$.

Statistical analyses Data are presented as means \pm 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 $9,727.9 \pm 2,629.8$ *Cacna1d* transcripts (Fig. 1a). Expression in INS-1 832/13 cells was similar to that observed in rat islets, with $1,580 \pm 337$ transcripts for *Cacna1c* and $4,266 \pm 749$ copies of *Cacna1d* (Fig. 1b). 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. 1c) compared with 19 ± 1 in beta cells alone (Fig. 1d). The number of *CACNA1D* mRNA molecules amounted to 550 ± 30 in islets (Fig. 1c) and $1,130 \pm 23$ in human beta cells (Fig. 1d), 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. 1i, k), but also in alpha cells (Fig. 1j).

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. 1e). *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. 1h). Interestingly, the expression of *CACNA1C* and *CACNA1D* showed strong covariation (Pearson's $r = 0.9$; $p = 2.5 \times 10^{-28}$; not shown). The

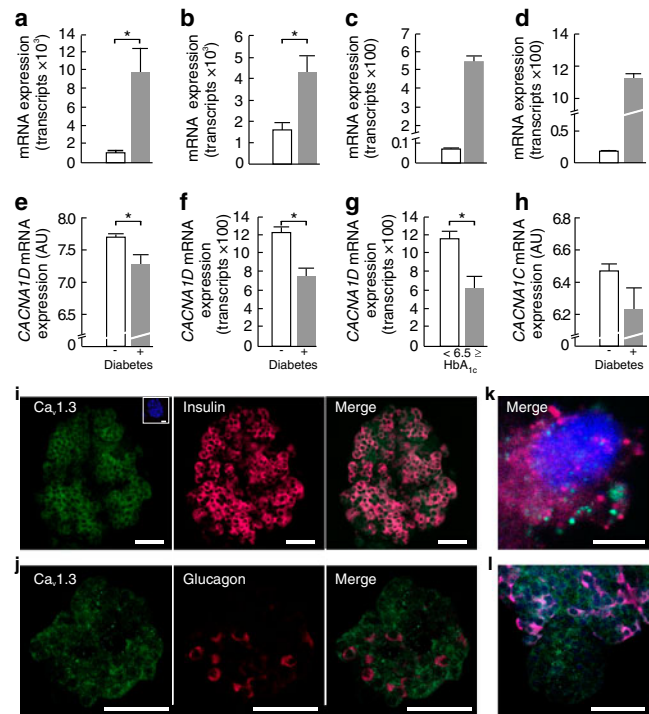


Fig. 1 Expression of *Cacna1c*/*CACNA1C* (encoding rat/human $Ca_v1.2$) and *Cacna1d*/*CACNA1D* (rat/human $Ca_v1.3$). (a, b) mRNA expression of *Cacna1d* (grey bar) exceeds that of *Cacna1c* (white bar) in rat islets (a) as well as in INS-1 832/13 cells (b). The same relationship, but with greater difference, 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} ($\geq 6.5\%$ [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 μ m. (k) A single cell showing distinct staining for insulin (red) and $Ca_v1.3$ (green). Scale bar 5 μ m. (l) Some small islets are $Ca_v1.3$ positive but lack insulin protein. Scale bar 50 μ m. All data are means \pm SEM. * $p < 0.05$. AU, arbitrary units

downregulation of *CACNA1D* in type 2 diabetes islets was confirmed with absolute qPCR ($1,235 \pm 64$ *CACNA1D* transcripts in 63 controls vs 754 ± 93 in seven cases; $p = 2 \times 10^{-4}$, *t* test; Fig. 1f). 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 $1,250 \pm 88$ transcripts when $HbA_{1c} < 6.5\%$ [48 mmol/mol]; $p = 0.008$, *t* test; Fig. 1g). We found no significant change in *INS* expression in type 2 diabetes islets as compared with controls ($2^{-\Delta Ct}$ *INS* relative to beta-actin $3.4 \times 10^{-5} \pm 1.5 \times 10^{-5}$ in cases vs

$4.6 \times 10^{-5} \pm 6.7 \times 10^{-6}$ 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 $\text{Ca}_v1.3 \text{ Ca}^{2+}$ channel gene *CACNA1D* that we observed in human pancreatic islets are also 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; $\beta=-0.103$; $p=0.036$, Table 2 and Fig. 2a). The

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

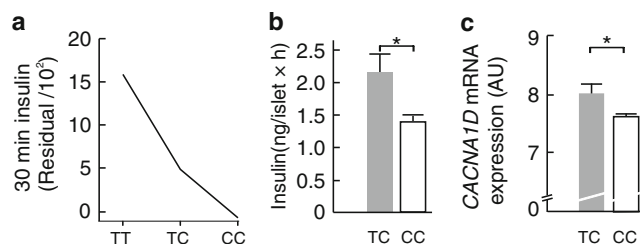


Fig. 2 (a) rs312480 has effects on in vivo and in vitro insulin levels and on mRNA expression. Thirty minute insulin levels (standardised residual corrected for age, sex and BMI shown) are reduced in rs312480/C allele carriers of the Botnia IVGTT Study (a) as well as in human islet batch incubations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

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 ($\beta=-0.047$; $p=0.048$) 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 \times h, $p=0.04$; T/T n.a.; Fig. 2b). 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. 2c).

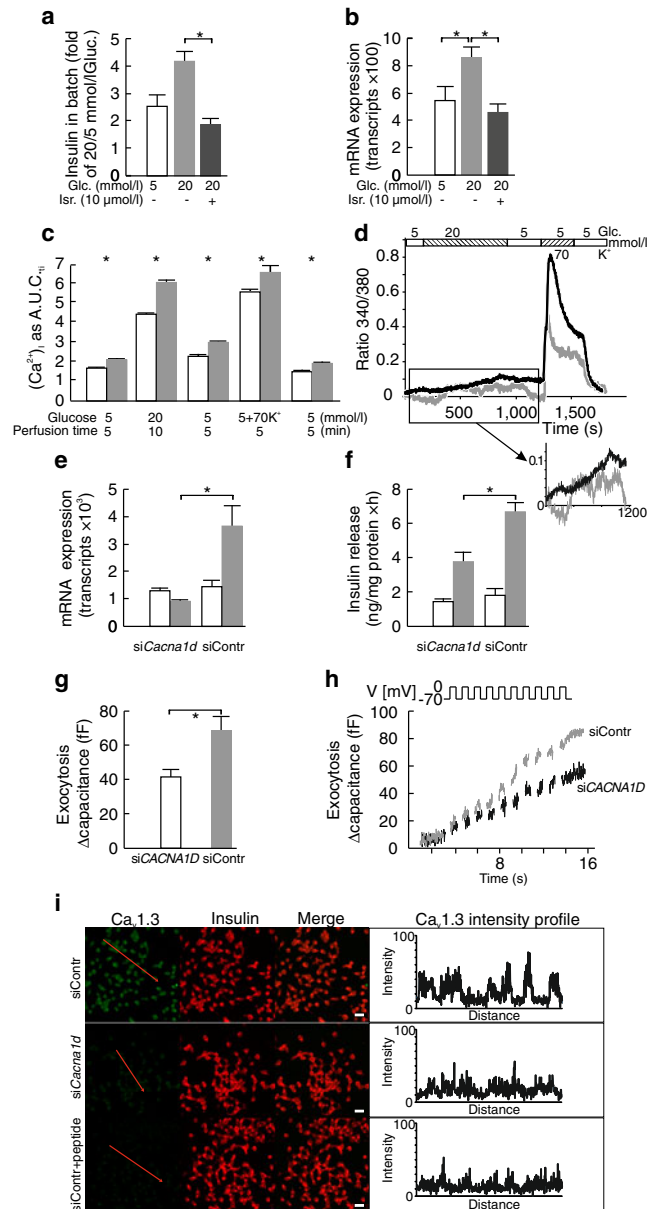
Neither the variant rs9841978 nor rs312486 showed a significant association with insulin secretion (ESM Table 3) or with changes in gene expression. However, we next wanted to examine if any of these SNPs in *CACNA1D* that were previously suggested to be associated with disease would also have an effect 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; $p=1.9 \times 10^{-4}$) 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 $p=1.1 \times 10^{-5}$) and

Fig. 3 *CACNA1D* expression changes upon incubation with 20 mmol/l glucose (Gluc.). 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 [A.U.C.₀₋₁₀] 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 $[\text{Ca}^{2+}]_i$ 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

for rs9841978/G the OR was 1.08 (95% CI 1.04, 1.12; $p = 1.1 \times 10^{-4}$). 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. 3a). 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. 3b). Increased *CACNA1D* expression coincided with elevated basal $[\text{Ca}^{2+}]_i$ 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; $p = 8 \times 10^{-4}$; Fig. 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; $p = 1 \times 10^{-5}$) 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; $p = 2 \times 10^{-3}$). Typical $[\text{Ca}^{2+}]_i$ oscillations during high-glucose stimulation were absent in the islets that had been incubated at 20 mmol/l glucose for 24 h (Fig. 3d). The elevated expression of *CACNA1D* by long-term 24 h glucose treatment is thus functionally important and clearly affects $[\text{Ca}^{2+}]_i$ homeostasis.

As we detected decreased *CACNAID* expression in the islets of individuals with type 2 diabetes, we wanted to examine whether the presence of *CACNAID* transcripts was a prerequisite for appropriate glucose-induced insulin release. We used siRNAs against human *CACNAID* (si*CACNAID*), 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 *CACNAID*, but both targeting rat *Cacna1d*) were comparably efficient and after 48 h *Cacna1d* expression in INS-1 832/13 cells was decreased by $72\pm 6\%$ (Fig. 3e, shown only for siRNA against rat *Cacna1d*; $p=0.02$) compared with the non-targeting siRNA. $\text{Ca}_v1.3$ protein was also decreased (Fig. 3i). We then tested for effects on GSIS. While basal release was not affected (Fig. 3f; 1.5 ± 0.1 ng insulin/mg protein \times h in si*Cacna1d*-treated vs 1.8 ± 0.3 ng insulin/mg protein \times h in control cells), the response to high glucose was significantly reduced in cells treated with si*Cacna1d* (Fig. 3f; 3.8 ± 0.4 ng/mg protein \times h in si*Cacna1d* vs 6.7 ± 0.7 ng/mg protein \times h in control; $p=0.0026$). Human beta cells responded in a similar manner. After 48 h incubation with si*CACNAID*, depolarisation-evoked single-cell exocytosis was significantly reduced when compared with control cells (Fig. 3g, h; 41 ± 4 fF in si*CACNAID* 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.

***CACNAID* and the exocytotic microdomain** Our data indicate that the appropriate expression of *CACNAID* and function of $\text{Ca}_v1.3$ are important determinants of GSIS. To further examine the role of $\text{Ca}_v1.3$ in the exocytotic process, we extended our microarray studies by exploring the genes that positively correlate with *CACNAID* expression (cut-off Pearson's $r \geq 0.8$, 163 genes). We found that genes coexpressed with *CACNAID* were significantly enriched for particular cellular functions (Table 3) and the top-ranked category was exocytosis ($p=9\times 10^{-4}$). This is in agreement with the functional results and underlines the relevance of these findings.

Table 3 Gene ontology analysis of the genes positively correlating with *CACNAID* 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	<i>p</i>
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

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 $\text{Ca}_v1.3$ dominates over the related $\text{Ca}_v1.2$ channel on the mRNA level, and that $\text{Ca}_v1.3$ mRNA and protein are mainly localised to human beta cells. Second, we have shown that a SNP (rs312480) in *CACNAID*, the gene encoding $\text{Ca}_v1.3$, 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 *CACNAID* mRNA is reduced in islets of patients with type 2 diabetes (Fig. 1) and that pharmacological or RNA interference-mediated inhibition of *CACNAID* expression and/or $\text{Ca}_v1.3$ 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 $\text{Ca}_v1.2$ [6, 7, 39, 40], with a diminished first phase after ablation of the channel [7]. However, a compensatory upregulation of $\text{Ca}_v1.2$ upon *Cacna1d* knockout, as detected by another group [8], could not be ruled out [39]. Studies in rats claimed $\text{Ca}_v1.3$ 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 $\text{Ca}_v1.3$ 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 *CACNAID* (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, $\text{Ca}_v1.3$, with its lower activation threshold compared with $\text{Ca}_v1.2$ [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], $\text{Ca}_v1.3$, with its low activation threshold, might be a good candidate for these events. In the glutamate-releasing inner-ear hair cell, $\text{Ca}_v1.3$ 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 $\text{Ca}_v1.3$ [8], although spontaneous spiking during this process has never been investigated. Reflecting on our finding that a polymorphism in the gene encoding for $\text{Ca}_v1.3$ 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. 3f, 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 *CACNAID* 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 *CACNAID* 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. 2a), *CACNAID* expression was no longer augmented (Fig. 2b). 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 *CACNAID* mRNA in the high-glucose condition back to isradipine levels (not shown). The observed glucose-induced activity-dependent rise in *CACNAID* expression correlated with increased basal Ca^{2+} levels, as well as higher levels under

stimulation (Fig. 3c, 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. 3d). 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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References

1. Rorsman P (1997) The pancreatic beta-cell as a fuel sensor: an electrophysiologist's viewpoint. *Diabetologia* 40:487–495
2. Davalli AM, Biancardi E, Pollo A et al (1996) Dihydropyridine-sensitive and -insensitive voltage-operated calcium channels participate in the control of glucose-induced insulin release from human pancreatic beta cells. *J Endocrinol* 150:195–203
3. Drews G, Krippeit-Drews P, Dufer M (2010) Electrophysiology of islet cells. *Adv Exp Med Biol* 654:115–163
4. Seisenberger C, Specht V, Welling A et al (2000) Functional embryonic cardiomyocytes after disruption of the L-type $\alpha 1C$ ($Ca_v1.2$) calcium channel gene in the mouse. *J Biol Chem* 275:39193–39199
5. Barg S, Eliasson L, Renstrom E, Rorsman P (2002) A subset of 50 secretory granules in close contact with L-type Ca^{2+} channels accounts for first-phase insulin secretion in mouse beta-cells. *Diabetes* 51(Suppl 1):S74–S82

6. Vignali S, Leiss V, Karl R, Hofmann F, Welling A (2006) Characterization of voltage-dependent sodium and calcium channels in mouse pancreatic A- and B-cells. *J Physiol* 572:691–706
7. Schulla V, Renstrom E, Feil R et al (2003) Impaired insulin secretion and glucose tolerance in beta cell-selective Ca(v)1.2 Ca²⁺ channel null mice. *EMBO J* 22:3844–3854
8. Namkung Y, Skrypnik N, Jeong MJ et al (2001) Requirement for the L-type Ca(2+) channel alpha1D subunit in postnatal pancreatic beta cell generation. *J Clin Invest* 108:1015–1022
9. Dreja T, Jovanovic Z, Rasche A et al (2009) Diet-induced gene expression of isolated pancreatic islets from a polygenic mouse model of the metabolic syndrome. *Diabetologia* 53:309–320
10. Koschak A, Reimer D, Huber I et al (2001) alpha 1D (Cav1.3) subunits can form I-type Ca²⁺ channels activating at negative voltages. *J Biol Chem* 276:22100–22106
11. Sinnegger-Brauns MJ, Huber IG, Koschak A et al (2009) Expression and 1,4-dihydropyridine-binding properties of brain L-type calcium channel isoforms. *Mol Pharmacol* 75:407–414
12. Seino S (1995) CACN4, the major alpha 1 subunit isoform of voltage-dependent calcium channels in pancreatic beta-cells: a minireview of current progress. *Diabetes Res Clin Pract* 28 (Suppl):S99–S103
13. Iwashima Y, Pugh W, Depaoli AM et al (1993) Expression of calcium channel mRNAs in rat pancreatic islets and downregulation after glucose infusion. *Diabetes* 42:948–955
14. Striessnig J, Bolz HJ, Koschak A (2010) Channelopathies in Ca(v)1.1, Ca(v)1.3, and Ca(v)1.4 voltage-gated L-type Ca(2+) channels. *Pflugers Arch* 460:361–374
15. Braun M, Ramracheya R, Bengtsson M et al (2008) Voltage-gated ion channels in human pancreatic beta-cells: electrophysiological characterization and role in insulin secretion. *Diabetes* 57:1618–1628
16. Yamada Y, Masuda K, Li Q et al (1995) The structures of the human calcium channel alpha 1 subunit (CACNL1A2) and beta subunit (CACNLB3) genes. *Genomics* 27:312–319
17. Yamada Y, Kuroe A, Li Q et al (2001) Genomic variation in pancreatic ion channel genes in Japanese type 2 diabetic patients. *Diabetes Metabol Res Rev* 17:213–216
18. Saxena R, Voight BF, Lyssenko V et al (2007) Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science* 316:1331–1336
19. Voight BF, Scott LJ, Steinthorsdottir V et al (2010) Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nat Genet* 42:579–589
20. Reinbothe TM, Ivarsson R, Li DQ et al (2009) Glutaredoxin-1 mediates NADPH-dependent stimulation of calcium-dependent insulin secretion. *Mol Endocrinol* 23:893–900
21. Lukowiak B, Vandewalle B, Riachy R et al (2001) Identification and purification of functional human beta-cells by a new specific zinc-fluorescent probe. *J Histochem Cytochem Offic J Histochem Soc* 49:519–528
22. Kirkpatrick CL, Marchetti P, Purrello F et al (2010) Type 2 diabetes susceptibility gene expression in normal or diabetic sorted human alpha and beta cells: correlations with age or BMI of islet donors. *PLoS One* 5:e11053
23. Jonsson A, Isomaa B, Tuomi T et al (2009) A variant in the KCNQ1 gene predicts future type 2 diabetes and mediates impaired insulin secretion. *Diabetes* 58:2409–2413
24. Lyssenko V, Eliasson L, Kotova O et al (2011) Pleiotropic effects of GIP on islet function involve osteopontin. *Diabetes* 60:2424–2433
25. Groop L, Forsblom C, Lehtovirta M et al (1996) Metabolic consequences of a family history of NIDDM (the Botnia study): evidence for sex-specific parental effects. *Diabetes* 45:1585–1593
26. Pyykkönen A-J, Räikkönen K, Tuomi T, Eriksson JG, Groop L, Isomaa B (2010) Stressful life events and the metabolic syndrome. *Diabetes Care* 33:378–384
27. Lindholm E, Agardh E, Tuomi T, Groop L, Agardh CD (2001) Classifying diabetes according to the new WHO clinical stages. *Eur J Epidemiol* 17:983–989
28. Berglund G, Elmstahl S, Janzon L, Larsson SA (1993) The Malmo Diet and Cancer study. Design and feasibility. *J Intern Med* 233:45–51
29. World Health Organisation (2006) Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF Consultation. Available from <http://www.who.int/entity/diabetes/publications/>. Accessed 11 June 2011
30. Pickering BM, Willis AE (2005) The implications of structured 5' untranslated regions on translation and disease. *Semin Cell Dev Biol* 16:39–47
31. Kamide K, Yang J, Matayoshi T et al (2009) Genetic polymorphisms of L-type calcium channel alpha1C and alpha1D subunit genes are associated with sensitivity to the antihypertensive effects of L-type dihydropyridine calcium-channel blockers. *Circ J* 73:732–740
32. Rosengren AH, Jokubka R, Tojjar D et al (2010) Overexpression of alpha2A-adrenergic receptors contributes to type 2 diabetes. *Science* 327:217–220
33. Taneera J, Jin Z, Jin Y, et al. (2012) γ -Aminobutyric acid (GABA) signalling in human pancreatic islets is altered in type 2 diabetes. *Diabetologia* 55:1985–1994
34. Taneera J, Lang S, Sharma A et al (2012) A systems genetics approach identifies genes and pathways for type 2 diabetes in human islets. *Cell Metabol* 16:122–134
35. Bigos KL, Mattay VS, Callicott JH et al (2010) Genetic variation in CACNA1C affects brain circuitries related to mental illness. *Arch Gen Psychiatry* 67:939–945
36. Sklar P, Smoller JW, Fan J et al (2008) Whole-genome association study of bipolar disorder. *Mol Psychiatr* 13:558–569
37. Green EK, Grozeva D, Jones I et al (2009) The bipolar disorder risk allele at CACNA1C also confers risk of recurrent major depression and of schizophrenia. *Mol Psychiatr* 15: 1016–1022
38. Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB (2000) Isolation of INS-1-derived cell lines with robust ATP-sensitive K⁺ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* 49:424–430
39. Platzer J, Engel J, Schrott-Fischer A et al (2000) Congenital deafness and sinoatrial node dysfunction in mice lacking class D L-type Ca²⁺ channels. *Cell* 102:89–97
40. Sinnegger-Brauns MJ, Hetzenauer A, Huber IG et al (2004) Isoform-specific regulation of mood behavior and pancreatic beta cell and cardiovascular function by L-type Ca²⁺ channels. *J Clin Invest* 113:1430–1439
41. Mangoni ME, Nargeot J (2008) Genesis and regulation of the heart automaticity. *Physiol Rev* 88:919–982
42. Comunanza V, Marcantoni A, Vandael DH et al (2011) CaV1.3 as pacemaker channels in adrenal chromaffin cells: specific role on exo- and endocytosis? *Channels* 4:440–446
43. Misler S, Dickey A, Barnett DW (2005) Maintenance of stimulus-secretion coupling and single beta-cell function in cryopreserved-thawed human islets of Langerhans. *Pflugers Arch* 450:395–404
44. Brandt A, Striessnig J, Moser T (2003) CaV1.3 channels are essential for development and presynaptic activity of cochlear inner hair cells. *J Neurosci* 23:10832–10840
45. Ahlgren U, Gotthardt M (2010) Approaches for imaging islets: recent advances and future prospects. *Adv Exp Med Biol* 654:39–57

46. Nitert MD, Nagorny CL, Wendt A, Eliasson L, Mulder H (2008) CaV1.2 rather than CaV1.3 is coupled to glucose-stimulated insulin secretion in INS-1 832/13 cells. *J Mol Endocrinol* 41:1–11
47. Hulme JT, Konoki K, Lin TW et al (2005) Sites of proteolytic processing and noncovalent association of the distal C-terminal domain of CaV1.1 channels in skeletal muscle. *Proc Natl Acad Sci U S A* 102:5274–5279
48. Satin J, Schroder EA, Crump SM (2011) L-type calcium channel auto-regulation of transcription. *Cell Calcium* 49:306–313
49. Bjorklund A, Lansner A, Grill VE (2000) Glucose-induced $[Ca^{2+}]_i$ abnormalities in human pancreatic islets: important role of overstimulation. *Diabetes* 49:1840–1848
50. Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL (2005) Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes* 54(Suppl 2):S97–S107