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Andersson, Sofia A; Olsson, Anders H; Esguerra, Jonathan L S; Heimann, Emilia; Ladenvall, Claes; Edlund, Anna; Salehi, S Albert; Taneera, Jalal; Degerman, Eva; Groop, Leif; Ling, Charlotte; Eliasson, Lena

Link to publication

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PO Box 117
221 00 Lund
+46 46-222 00 00

Title: Reduced insulin secretion correlates with decreased expression of exocytotic genes in pancreatic islets from patients with type 2 diabetes

Sofia A Andersson[1], Anders H Olsson[2], Jonathan LS Esguerra[1], Emilia Heimann[5], Claes Ladenvall[3], Anna Edlund[1], Albert Salehi[4], Jalal Taneera[3], Eva Degerman[5], Leif Groop[3], Charlotte Ling[2] and Lena Eliasson[1]*

[1]Islet Cell Exocytosis, [2] Epigenetics and Diabetes, [3] Diabetes and Endocrinology and [4]Islet Cell Physiology, Lund University Diabetes Centre, Department of Clinical Sciences Malmö, Lund University, Malmö, Sweden [5]Department of Experimental Medical Sciences, Biomedical Center, Lund University, Lund, Sweden.

*Address for correspondence: Lena Eliasson, Islet Cell Exocytosis, Lund University Diabetes Centre, CRC 91-11, SUS Malmö entrance 72, 205 02 Malmö, Sweden, phone: +46 40 391153, fax +46 40 391222, email: lena.eliasson@med.lu.se

Running title: Exocytotic genes in type 2 diabetic human islets

Word count (abstract/main text): 148/5471

*Abbreviations***:**

T2D: Type 2 diabetes

GSIS: Glucose-stimulated insulin secretion

SNP: Polymorphism

DGI: Diabetes Genetic Initiative

BIS: Basal insulin secretion

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Abstract

Reduced insulin release has been linked to defect exocytosis in β-cells. However, whether expression of genes suggested to be involved in the exocytotic process (exocytotic genes) is altered in pancreatic islets from patients with type 2 diabetes (T2D), and correlate to insulin secretion, needs to be further investigated.

Analysing expression levels of 23 exocytotic genes using microarray revealed reduced expression of five genes in human T2D islets (χ^2=13.25; p<0.001). Gene expression of *STX1A, SYT4, SYT7, SYT11, SYT13, SNAP25* and *STXBP1* correlated negatively to *in vivo* measurements of HbA1c levels and positively to glucose stimulated insulin secretion (GSIS) *in vitro* in human islets. STX1A, SYT4 and SYT 11 protein levels correspondingly decreased in human T2D islets. Moreover, silencing of SYT4 and SYT13 reduced GSIS in INS1-832/13 cells. Our data support that reduced expression of exocytotic genes contributes to impaired insulin secretion, and suggest decreased expression of these genes as part of T2D pathogenesis.

1. Introduction

Type 2 diabetes (T2D) is characterized by increased blood glucose levels, due to both reduced insulin secretion from the pancreatic β-cell, and impaired insulin action at the target cells. Several studies have shown that impaired insulin secretion occurs already before the onset of T2D (Del Prato and Tiengo, 2001,Gerich, 2002,Hosker, Rudenski, Burnett et al., 1989) signifying that processes involved in insulin secretion are crucial in the development of the disease.

Insulin release is biphasic with a first rapid phase followed by a second slower phase. Patients with T2D characteristically display reduced or complete absence of the first phase (Del Prato and Tiengo, 2001,Gerich, 2002,Hosker et al., 1989), which is suggestive of impaired priming and/or fusion of the insulin-containing granules with the plasma membrane (Barg, Eliasson, Renstrom et al., 2002). Priming and fusion are processes involved in the cascade of events that precedes Ca^{2+}-dependent exocytosis and the release of insulin (Eliasson, Abdulkader, Braun et al., 2008). During the priming process the SNARE complex assemble to prepare the granules for Ca^{2+}-dependent fusion. In short, the plasma membrane associated proteins syntaxin 1A (Stx1A) and synaptosomal-protein of 25kD (Snap25) interact with vesicle-associated membrane protein (Vamp2), which gives rise to a tertiary SNARE complex that promotes fusion by pulling the vesicle membrane in close contact with the plasma membrane upon Ca^{2+} influx (Gerber and Sudhof, 2002).

The importance of the SNARE complex for insulin secretion in β-cells has been stated in several studies (reviews e.g. (Eliasson et al., 2008,Leung, Kwan, Ng et al., 2007,Wang and Thurmond, 2009)). Stx1A proteins are arranged in clusters along the plasma membrane in close association with the insulin granules and the number of clusters are reduced in the GK-rat (Ohara-Imaizumi, Nishiwaki, Nakamichi et al., 2004). In accordance, the diabetic GK-rat has reduced levels of *stx1A* (Gaisano, Ostenson, Sheu et al., 2002). Inhibition of Stx1 in mice upon addition of an antibody reduces Ca^{2+}-dependent insulin secretion (Martin, Moya, Gutierrez et al., 1995) and decreases the exocytotic response by 90%, an effect that is strongly associated with inhibition of the Ca^{2+}current (Vikman, Ma, Hockerman et al., 2006).

The Ca^{2+}-sensing element in regulated exocytosis is proposed to be aided by some isoforms in the family of synaptotagmins (Syts;(Pang and Sudhof, 2010)), namely Syt1-3, 5-7 and 9-10. These isoforms display Ca^{2+}-dependent phospholipid binding to the C_2A-domain, whereas the other members do not. The family of synaptotagmins is comprised of at least16 members out of which Syt1-6 and 9-13 has previously been shown to be expressed in brain tissue (Pang and Sudhof, 2010,Sudhof, 2002) where Syt5, Syt9 and *Syt13* have also been detected in primary β-cells (Grise, Taib, Monterrat et al., 2007,Iezzi, Kouri, Fukuda et al., 2004,Parton, McMillen, Shen et al., 2006) and *Syt4* transcript has been shown to be expressed in clonal β-cell lines as well as in islet cells (Gao, Reavey-Cantwell, Young et al., 2000,Gut, Kiraly, Fukuda et al.,

2001). Syt7, 8, 14 and 15 are mainly expressed in heart, kidney, and pancreas (Gauthier and Wollheim, 2008). The effect of Syt7 has previously been studied in Syt7 knockout mice, which display impaired glucose tolerance and lowered basal- and glucose-induced insulin levels (Gustavsson, Lao, Maximov et al., 2008).

Apart from Stx1A, Snap25, Vamp2 and the Syts, successful β-cell exocytosis involve several proteins such as Rim1, Rim2, Stxbp (or Munc-18) and Unc-13 (or Munc-13) (Gandini, Sandoval, Gonzalez-Ramirez et al., 2011,Kwan, Xie, Sheu et al., 2006,Yasuda, Shibasaki, Minami et al., 2010,Zhang, Efanov, Yang et al., 2000). Most of the published work on exocytotic proteins and insulin secretion has investigated the functional role of these proteins in rodents and less has been performed in human islets or in genetic studies. One previous study performed on islets from 8 donors demonstrated that expression of *STX1A*, *SNAP25*, *VAMP2* and *STXBP* is down-regulated in donors with T2D compared to non-diabetic controls (Ostenson, Gaisano, Sheu et al., 2006). Transcripts of *STX7, SYT5* and *SYT1* in islets from a donor with T2D and expression of *Syt13* in Wistar rats have been demonstrated to be up-regulated by palmitate (Ghanaat-Pour and Sjoholm, 2009,Ghanaat-Pour and Sjoholm, 2011).

T2D is a complex disease most likely comprised of both environmental and genetic factors where in the case of heritability, effects seem to be more pronounced on insulin secretion in the pancreatic β-cells than on insulin action in the target tissue. (Lehtovirta, Kaprio, Forsblom et al., 2000). Interestingly, many of the identified polymorphisms associated with T2D are related to β-cell function (Koeck, Olsson, Nitert et al., 2011,Lyssenko, Lupi, Marchetti et al., 2007,Olsson, Ronn, Ladenvall et al., 2011,Rosengren, Jokubka, Tojjar et al., 2010) e.g. two studies link SNPs in *STX1A* to T2D (Tsunoda, Sanke, Nakagawa et al., 2001,Romeo, Sentinelli, Cavallo et al., 2008). Genome-Wide Association Studies (GWAS) offer the possibility to explore single nucleotide polymorphisms (SNPs) in the genome that may be of importance in T2D (Saxena, Voight, Lyssenko et al., 2007). The GWAS Diabetes Genetics Initiative (DGI) provides information of SNP associations with 18 quantitative traits, including measures of insulin and glucose levels during an oral glucose tolerance test (OGTT).

Taken together, previous reports suggest that defects in the exocytotic machinery may contribute to the pathogenesis of T2D. For this reason, we have chosen to closer evaluate the expression levels of 23 selected genes associated with the exocytotic process in pancreatic islets from human donors with or without T2D. We propose that mRNA expression of exocytotic genes is altered in islets from T2D donors, and furthermore that exocytotic gene expression correlates with insulin secretion in human islets. In addition, we aim to examine if there are SNPs in or near exocytotic genes that correlate to phenotypes associated with impaired insulin secretion, and/or with gene expression itself.

2. Materials and methods

2.1. Human pancreatic islets

Human pancreatic islets from 55 non-diabetic and 9 T2D deceased donors were used for microarray analysis, qPCR and glucose stimulated insulin secretion (Table 1). Additional islets from 4 non-diabetic and 4 T2D donors were used for measurements of protein levels. All human islets were obtained through collaboration between the Human Tissue Laboratory at Lund University Diabetes Centre and the Nordic Network for Clinical Islet Transplantation headed by Prof. Olle Korsgren at Uppsala University, Sweden. The donors had given their consent before death, or consent was given by her/his relatives upon admission to Intensive Care Unit (ICU,) to donate organs and the protocols were approved by the local ethics committees. Islets were prepared by collagenase digestion and density gradient purification. After isolation, islets were cultured1-7 days, free floating in CMRL 1066 culture medium (ICN Biomedicals, Costa Mesa, CA, USA) containing human serum and 5.56 mM glucose and supplemented with 10 mmol/l HEPES, 2 mmol/l 1-glutamine, 50 µg/ml gentamicin, 0.25 µg/ml Fungizone (GIBCO, BRL, Gaithersburg, MD, USA), 20 µg/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany), and 10 mmol/l nicotinamide at 37°C (5% CO_2) prior to RNA and DNA preparation. The preparation of islets can be contaminated by exocrine tissue and is often therefore not 100% pure. However, the islet purity was similar for non-diabetic and T2D donors (68±18% *vs* 58±19%, p=0.15) using dithizone staining. We further used gene expression of endocrine (somatostatin and glucagon) and exocrine (pancreatic lipase, α2A amylase, and chymotrypsin 2) markers to determine islet purity, and again there was no difference in purity between non-diabetic and T2D donors (72 *vs* 68%, p=0.29) (Olsson, Yang, Hall et al., 2011). It was controlled that days in culture was not affecting the expression of the investigated genes. Insulin secretion measurements were performed on hand-picked islets, which obviously increased the purity.

2.2 Animal Model

The Sprague Dawley rat was purchased from Charles River Laboratories (Germany) and kept under standardized conditions in the animal house facilities. The experimental procedure has been approved by the Committee of ethical animal research in Malmö and Lund (permission number: M249-11). Rat brain was used as control tissue in the western blot analysis.

2.3. Glucose-stimulated insulin secretion in human islets

Islets were hand-picked under a stereomicroscope at room temperature and preincubated for 30 min at 37°C in Krebs Ringer bicarbonate (KRB) buffer (pH 7.4) containing (in mM) 120 NaCl, 25NaHCO$_3$, 4.7 KCl, 1.2 MgSO$_4$, 2.5 CaCl$_2$, 1.2 KH$_2$PO, 10 HEPES supplemented with 1 mg/ml

albumin, N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid (10 mM) and 1 mM glucose. Each incubation vial contained 12 size-matched islets in 1.0 ml KRB buffer solution and was treated with 95% O_2-5% CO_2 to obtain constant pH and oxygenation. After preincubation, the buffer was changed to a KRB buffer containing either 1 mM (basal secretion; BIS) or 16.7 mM glucose (stimulated secretion; GSIS). The islets were then incubated for 1h at 37°C in a metabolic shaker (30 cycles per min). Immediately after incubation an aliquot of the medium was removed for analysis of insulin using a radioimmunoassay kit (Euro-Diagnostica, Malmö, Sweden). For each individual, measurements were performed in 6-8 vials per condition.

2.4. Gene expression in human pancreatic islets

Total RNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Hilden, Germany). RNA quality and concentration was measured using an Agilent 2100 bioanalyzer and Nanodrop ND-1000 equipment, respectively.

The microarrays were performed following the Affymetrix standard protocol. Briefly, 200 ng of total RNA was processed following the GeneChip® Expression 3'-Amplification Reagents One-cycle cDNA synthesis kit instructions (Affymetrix Inc, Santa Clara, CA, US to produce double-stranded cDNA. This was used as a template to generate biotin-targeted cRNA following manufacturer's specifications. 15 µg of the biotin labeled cRNA was fragmented to strands between 35 and 200 bases in length, 10 µg of which was hybridized onto the GeneChip® Human Gene 1.0 ST whole transcript based assay overnight in the GeneChip® Hybridization oven 6400 using standard procedures. The arrays were washed and stained in a GeneChip® Fluidics Station 450. Scanning was carried out with the GeneChip® Scanner 3000 and image analysis was performed using GeneChip® Operating Software. The array data was summarized and normalized with Robust Multi-array Analysis (RMA) method using the software "Expression Console" (Affymetrix). In the present study, we studied the expression of 23 selected exocytotic genes using data from the microarray. The genes were selected based on their previously known function in exocytosis in pancreatic islets (Eliasson et al., 2008,Gauthier and Wollheim, 2008).

2.5. Quantitative RT-PCR

Quantitative real time PCR (qRT-PCR) was performed for selected genes in order to technically validate the results obtained in the microarray study. Total RNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Hilden, Germany). RNA quality and concentration was measured using an Agilent 2100 bioanalyzer and Nanodrop ND-1000 equipment, respectively. cDNA was synthesized using QuantiTect Reverse Transcription kit (Qiagen GmbH, Hilden, Germany). Quantitative analysis of gene expression was performed using the TaqMan qRT-PCR with an ABI Prism 7900 HT System (Applied Biosystems, USA) using gene-specific probes and

primer pairs (Assays-on-demand, Applied Biosystems, USA) for *SYT4* (assay ID: Hs01086433_m1), *SYT7* (Hs01590513_m1) and *STX1A* (Hs00270282_m1) in triplicates on 384-well plate format using ABI 7900 platform (Applied Biosystems, USA). 1.2 ng cDNA was used per well in 10-µL reaction volume containing TaqMan master mix (Applied Biosystems, USA). No template control (NTC) wells containing aliquots from the RT reaction without the reverse transcriptase enzyme were included to control for genomic DNA contamination. The human HPRT1 (Applied Biosystem, USA) exhibited stable expression in all islet RNA preparations and was used to normalize gene expression by the ΔΔCt method, where the final normalized quantity was expressed as 2^-(Ct target - Ct control).

2.6. Immunocytochemistry.

Handpicked human islets were transferred to calcium free isolation solution and dispersed into single cells. Cells were seeded on poly-L-leucine (Sigma-Aldrich) coated dishes and cultured overnight in RPMI-1640 medium (SVA, Sweden) supplemented with 10 mM glucose, 5% (vol/vol) fetal calf serum, 100 µg/ml streptomycin, 100 IU/ml penicillin and 2 mM L-Glutamine (Sigma-Aldrich). Cells were fixed and stained as described elsewhere (Vikman et al., 2006). Primary antibodies rabbit polyclonal anti-Syntaxin1A (SySy 110 302) and Guinea pig monoclonal anti-insulin (EuroDiagnostica B65-1) was used and secondary antibodies for rabbit conjugated to Cy5 (Jacksson 711:175:152) and Guinea-pig conjugated to Cy2 (Jackson 706:225:148). Immunoflourescent was detected using confocal microscopy (META 510, Carl Zeiss, Germany). Mean intensity of fluorescence was analysed in Zen 2009 version software (Carl Zeiss, Germany). The plasma membrane and cytosol was marked and the nucleus excluded. Mean intensity of each cell were divided by the area of the cell.

2.7. Western blot analysis

Frozen human pancreatic islets were thawed, resuspended in a buffer containing 50 mM TES, pH 7.4, 250 mM sucrose, 1 mM EDTA, and 0.1 mM EGTA, supplemented with Complete Protease Inhibitor Cocktail (Roche) and homogenized by 10 short sonication pulses. Whole rat brain (including cerebellum) was resuspended in a buffer containing 50 mM TES, 2 mM EDTA, 0.1 mM EGTA, 250 mM Sucrose, 40 mM phenylphosphate, 5 mM NaF, 1 mM dithioerythriol, 0.5 mM sodium orthovanadate, 10 µg/mL antipain, 10 µg/mL leupeptin, 1µg/mL pepstatin, pH 7.4 at a ratio of 3 mL buffer per gram of tissue and homogenized with a large glass homogenizer. Total protein amount was determined according to Bradford (Bradford, 1976). Homogenates from human pancreatic islets and rat brain were subjected to SDS-PAGE. Proteins were transferred to polyvinylidene membranes (Millipore) and the membranes were stained with Ponceau S (0.1% in 5% acetic acid) and blocked with 10% milk in a buffer consisting of 20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.1% (v/w) Tween-20 for 60 min. Membranes with

proteins were probed with antibodies for SYT4 (Synaptic Systems), SYT7 (Synaptic Systems), SYT11 (Sigma-Aldrich), SYT13 (Nordic BioSite) and Cyclophilin B (PPIB) (Abcam) and incubated overnight at 4°C. Proteins were detected using the chemiluminescent Super Signal West Pico Luminol/Enhancer solution from Pierce and the ChemiDoc XRS+ Imager (Bio-Rad Laboratories). Quantification was made using Image Lab™ Software (Bio-Rad Laboratories).

2.8. siRNA transfection and insulin secretion assay on INS1-832/13 cells

INS1-832/13 cells were seeded at density of 400,000 cells per well on a 24-well plate in 1 mL RPMI medium complete supplement without antibiotics. The following day, the cells were transfected with *Silencer® Select Pre-Designed siRNA* (LifeTechnologies), siRNA ID in bracket: Stx1A (s137946 and s137944), Syt4 (s134069), Syt7 (s133151), Syt11 (s133472), Syt13 (s135284) and Negative control No.1 siRNA (Cat# 4390844) using Lipofectamine RNAiMAx (Invitrogen). A final transfection volume of 600 µL per well contain 1.5 µL of transfection reagent and 50 nM of siRNA in Opti-MEM reduced serum media (Invitrogen). After 4 hours, 500 µL of RPMI medium with complete supplement without antibiotics was added. Medium was changed the following the day and the cells were assayed after 48 h for insulin secretion with subsequent protein and RNA extraction. Knockdown was assessed by RT-qPCR of the target genes as described above using the following Taqman® gene expression assays (Life Technologies): Stx1A (Rn00587278_m1), Syt4 (Rn01157571_m1), Syt7(Rn00572234_m1), Syt11(Rn00581475_m1) and Syt13 (Rn00578161_m1).

After transfection insulin secretion measurements were performed. Confluent plates containing transfected INS1-832/13 cells were washed twice with 1 mL pre-warmed Secretion Assay Buffer (SAB), pH 7.2 (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 25.5 mM $NaHCO_3$ and 0.2% Bovine Serum Albumin) containing 2.8 mM glucose. The cells were then pre-incubated for two hours in new 2 mL SAB with 2.8 mM glucose. Afterwards, separate wells were incubated for 1 h in 1 mL SAB containing either 2.8 mM or 16.7 mM glucose. Secreted insulin was measured from supernatant using Coat-a-Count Insulin radioimmunoassay kit (Siemens) and the values were normalized using total protein content individually for each well.

2.9. Study population

The Diabetes Genetic Initiative (DGI) is a case-control study of individuals with T2D and matched non-diabetic controls from Finland and Sweden (Saxena et al., 2007). 1463 patients were classified with T2D according to WHO (1998) criteria with fasting plasma glucose ≥7.0 mmol/l or a 2 h glucose ≥11.1 mmol/l during an oral glucose tolerance test (OGTT). The 1467 control subjects were defined as normal glucose tolerant; with fasting plasma glucose <6.1

mmol/l and 2 h glucose <7.8 mmol/l, and had no first degree relatives with T2D (Table S1; (Saxena et al., 2007)). In the present study, we included non-diabetic subjects only. Measurements of insulin and glucose were performed according to the description of DGI at the Broad Institute homepage (http://www.broadinstitute.org/diabetes). Insulinogenic index was calculated as $(Insulin_{30}-Insulin_{0})/(Glucose_{30}-Glucose_{0})$.

The Prevalence, Prediction and Prevention of Diabetes (PPP-Botnia), a population based study in the Botnia region of Western Finland (Pyykkonen, Raikkonen, Tuomi et al., 2010) was used as a replication cohort for genetic associations. Diagnosis of T2D was confirmed from subject records or on the basis of fasting plasma glucose concentration ≥7.0 mmol/l and/or 2 h glucose ≥11.1 mmol/l). 4323 non-diabetic subjects were included in this study (Table S1).

All participants in DGI and PPP-Botnia gave written informed consent for the studies and the local ethics committees approved the protocols.

2.10. Genotyping

In the DGI GWAS, genotyping was performed using Affymetrix 500K chip array (Saxena et al., 2007). In PPP-Botnia and in human islets from cadaver donors rs12524194 and rs1339226 were genotyped using allelic discrimination assays on the ABI 7900 platform (KASP on Demand, KBioscience, Hertfordshire, UK and C__8331634_10, Applied Biosystems, Foster City, CA, respectively). The genotyping call rates for both assays were above 96% and genotype calls were in concordance based on 4.2% duplicate comparisons.

2.11. Statistical analyses

The mRNA expression of the 23 exocytotic genes on the microarray was compared between T2D and non-diabetic donors using Mann-Whitney U-tests.

A χ^2-test was used to analyze if a set of exocytotic genes showed significantly reduced gene expression in pancreatic islets from individuals with T2D compared with non-diabetic pancreatic islets. The test compared the observed (n=5) and expected (n=1.13) number of exocytotic genes with nominally differential gene expression (p≤0.05) The expected number of exocytotic genes was calculated based on the number of analyzed exocytotic genes using the microarray (n=23), the total number of probeIDs analyzed using the microarray (n=28869) and the total number of probeIDs that showed nominally reduced gene expression in the T2D human islets (n=1416).

Correlations were calculated using Spearman correlation coefficient (ρ).

Differences between groups in insulin secretion measurements performed in INS1-832/13 cells after transfection with different siRNA were tested using Student's t-test and multiple comparisons were corrected using the Holm-Bonferroni method.

Genotype-phenotype associations were analyzed using linear regression analyses adjusted for age, gender, BMI (DGI, PPP-Botnia) and geographic origin (DGI) assuming an additive genetic model. Clinical characteristics of the participants in DGI and PPP-Botnia (Table S1) are expressed as mean ± s.d or median (IQR). A genomic control inflation factor was used to correct for population stratification (DGI). Phenotype values were logarithmically transformed to better fit a normal distribution in both cohorts before analyses. Results are presented as beta coefficient ± SEM.

Statistical calculations were performed using PASW Statistics 18 for Windows (SPSS, Chicago, IL, USA). A p-value less than 0.05 was considered significant.

3. Results

3.1. Decreased expression of genes involved in exocytosis in human islets from T2D donors

We were interested to examine if gene expression of exocytotic genes is down-regulated in human islets from patients with T2D. Therefore, we analyzed microarray data of human pancreatic islets from 55 non-diabetic and 9 T2D donors (Table 1). We identified 5 genes with nominally reduced expression in islets from T2D donors compared to non-diabetic donors (Fig. 1) namely, *STX1A* (p= 0.010)*, SYT4* (p=0.017)*, SYT7* (p=0.018)*, SYT11* (p=0.041) and *SYT13* (p=0.0033). A χ^2- test was next performed to examine if the observed number of exocytotic genes down-regulated in T2D islets is more than expected. The frequency of observed exocytotic genes with reduced expression (n=5) was compared to the theoretically expected number of down-regulated exocytotic genes (n=1.13) in islets from T2D compared to non-diabetic donors. This resulted in a χ^2-value of observed over theoretically expected down-regulated exocytotic genes of 13.25 with p<0.001.

To validate the down-regulation seen in the microarray, 3 genes were selected for technical replication using qRT-PCR. The technical validation (Fig. 2), ensued the reduced expression levels of *STX1A* (p=0.027), *SYT4* (p=0.017) and *SYT7* (p=0.014) in human islets from T2D donors (N=7) compared to non-diabetic donors (N=47).

Confirmation that the reduced gene expression could be transferred to protein level was performed using confocal immunocytochemistry and western blot analysis. The protein level of STX1A was significantly reduced in human islet from a T2D donor (n=14) as compared to the level in islets from non-diabetic donors (N=2, n=37); Fig. 3a and b). Furthermore, western blot analysis (Fig. 3c and d) revealed a significant decrease in the protein level of SYT4 and SYT11 in islets from T2D donors (N=2) as compared to non-diabetic donors (N=3). SYT7 protein level was reduced by ~50% compared to mean control levels in one of the islet samples from a T2D donor (Fig. 3c), but not in the other two investigated samples, and in summary SYT7 protein levels showed no significant reduction (Fig. 3d). There was no significant difference in protein levels of SYT13.

3.2. Correlation between expression of genes involved in exocytosis to GSIS and HbA1c

Exocytosis and thereby insulin secretion is dependent on the expression of exocytotic genes, and studies in rodents have demonstrated impaired insulin secretion and exocytosis upon ablation of these genes (see e.g. (Leung et al., 2007,Wang and Thurmond, 2009,Gustavsson et al., 2008)). Thus, a positive correlation of mRNA expression of the exocytotic genes to GSIS suggests that exocytotic genes may promote insulin secretion. Indeed, we found positive correlations between mRNA expression and GSIS for *STX1A* (p=0.004), *SYT4* (p=0.006), *SYT7* (p=0.039) as well as *SNAP25* (p=0.042), *STXBP1* (p=0.038), *SYT11* (p=0.013) and *SYT13* (p=0.002) using data from

all donors combined (Table 2). When analyzing data from non-diabetic donors alone similar trends of ρ were obtained (Table S2) although it did not always reach significance. The respective trend lines are illustrated for *STX1A* and *SYT13* in Fig. 4 a and b. There was no correlation between the expression levels and basal insulin secretion (BIS), suggesting that the exocytotic genes are more important for fuel-stimulated and regulated Ca^{2+}-dependent exocytosis.

Furthermore, when correlating the gene expression of these exocytotic genes with HbA1c levels, a well-known measure of glucose homeostasis and a long term indicator of blood glucose levels, negative correlations were obtained for *STX1A* (p=0.005), *SYT4* (p=0.006), *SYT7* (p=0.022), *SNAP25* (p=0.018), *STXBP1* (p=0.007), *SYT11* (p=0.052) and *SYT13* (p=0.001) (Table 2). Again, the trends were comparable when including or excluding the data from T2D donors (Fig. 4c and d, Table S2). This is in agreement with the hypothesis that hyperglycemia seen in T2D is partly due to impaired insulin secretion (Eliasson et al., 2008,Wang and Thurmond, 2009).

3.3. Silencing of exocytotic genes reduce insulin secretion

In order to mechanistically investigate if reduced expression of exocytotic genes can be transferred to an effect on insulin secretion we performed experiments on INS1-832/13 cells transfected with siRNA against Stx1A, Syt4, Syt7, Syt11 and Syt13 and a scramble control. The treatment reduced the gene expression of the selected genes by ~50-70% as compared to scramble control (Fig. 5a). SiRNA silencing of Syt4 and Syt13 significantly reduced insulin secretion after incubation in 16.7 mM glucose for one hour (Fig. 5b). Silencing of Syt7 reduced GSIS (p=0.036 *vs* scramble control), but the differences in mean-values did not reach significance following Holm-Bonferroni correction. Furthermore, there was no reduction in GSIS after silencing of Stx1A and Syt11. The lack of effect following siRNA silencing of Stx1A was surprising, why we tested a second siRNA, which showed the same result (data not shown).

3.4. Polymorphisms in the *RIMS1* gene is associated with impaired insulin secretion

We further extracted data from the DGI study to examine if there are SNPs in or near the 23 selected exocytotic genes that are associated with insulin and/or glucose measurements during OGTTs in the non-diabetic individuals of DGI (Table S1). A region 10 kb upstream and downstream of each gene was included in the analysis. In DGI, 19 of the 23 selected genes were available for SNP analysis (Table S3). Nominal associations were detected between SNPs in exocytotic genes and the following phenotypes; fasting glucose, 2-h glucose, fasting insulin, 2h-insulin, and/or insulinogenic index. We hence selected SNPs of exocytotic genes that were nominally associated with at least two of these phenotypes (requiring p-values ≤0.01 and Minor Allele Frequency (MAF) ≥ 0.05; Table S4). The top candidate SNPs, based on strongest associations, was rs12524194 and rs1339226 in *RIMS1*. These SNPs were associated with 2-h glucose and insulinogenic index. The beta-values were positive for both 2-h glucose and insulinogenic index (Table S4). The positive beta-coefficient for insulinogenic index indicates that the major allele associate with reduced insulin secretion. The associations between the two selected SNPs in *RIMS1* and 2-h glucose and insulinogenic index could not be replicated in the PPP-Botnia study. However, the G-allele of rs12524194 was associated with reduced expression of *RIMS1* (p=0.045) in human islets from non-diabetic donors (Fig. 6).

4. Discussion

A typical feature of T2D is a loss of the first phase of the biphasic insulin secretion. It is suggested that the loss correlates to an impairment of the machinery underlying the exocytotic process of insulin release, and we hence hypothesized that the mRNA expression of exocytotic genes is altered in T2D islets. Indeed, we found that expression of *STX1A*, *SYT4, SYT7, SYT11* and *SYT13* was nominally down-regulated in islets from T2D donors. In addition, the expression of these genes correlated positively with GSIS and negatively with HbA1c levels, supporting the importance of exocytotic genes for insulin secretion and glucose homeostasis.

In agreement with a previous study (Ostenson et al., 2006) we demonstrate down-regulation of *STX1A* gene expression in islets from T2D donors, and that expression of *STXBP1* and *SNAP25* negatively correlate with HbA1c levels. Furthermore, we identified several novel members belonging to the *SYT* family i.e. *SYT4*, *7*, *11* and *13*, which exhibit decreased expression in islets from T2D donors. Of the five genes with reduced expression, three was demonstrated to have reduced protein levels; STX1A, SYT4 and SYT11 in islets from T2D donors. Protein levels were investigated in a smaller number of individuals which might be reflected in that SYT7 and SYT13 protein levels were not significantly different. The disparities seen might be due to the inter-individual differences within the studies in terms of number of individuals investigated, the matching of the two groups and the clinical differences between individuals. Regardless, it highlights that reduced expression of exocytotic genes may be an important factor involved in T2D. It should be acknowledged that the mean BMI of the T2D donors in this study was fairly low (Table 1), which may suggest that some of the donors were undiagnosed with T1D. However, a high BMI is not the sole cause of T2D and we have recently published data where we demonstrate that a set of genetic variants is associated with reduced exocytosis and that this effect is more pronounced in islets from donors with a low BMI (Rosengren, Braun, Mahdi et al., 2012). The reduced expression of exocytotic genes is most likely not due to altered proportions of the different cell types of the human islets since it has been reported that the β-cell number is not reduced in T2D (Clark, Jones, de Koning et al., 2001,Rahier, Goebbels and Henquin, 1983) although this cannot be fully discarded (Butler, Janson, Bonner-Weir et al., 2003).

It has previously been demonstrated that immunoneutralization of Stx1 (Martin et al., 1995,Vikman et al., 2006) or expression of botulinum neurotoxin C1 light chain to cleave Stx1 (Lang, Zhang, Vaidyanathan et al., 1997) result in reduced exocytosis. Furthermore, studies in mice depleted of Stx1 display decreased number of docked granules at the plasma membrane and absence of first phase insulin secretion (Ohara-Imaizumi, Fujiwara, Nakamichi et al., 2007). Indeed, in our study, expression of *STX1A* in human islets correlated positively with GSIS (Fig. 4a). Surprisingly, we observed no effects on GSIS following siRNA silencing of Stx1A in INS1-832/13 cells (Fig. 5b). However, the siRNA secretion-experiments performed over one hour in high glucose is dominated by the second phase of insulin secretion, whereas Stx1A most likely influence first phase insulin secretion. Thus, effects on the first phase insulin secretion might not

be detected. Apart from Stx1A, Stx4 has been suggested to be important for insulin secretion in rodents. Stx4 has been demonstrated to be more important for second phase insulin secretion (Jewell, Oh and Thurmond, 2010). Thus it might be that Stx4 oversees second phase insulin secretion and can compensate for the loss of Stx1, although it was ascertained that Stx4 mRNA levels was not changed after siRNA silencing of stx1A (data not shown). Another explanation could be that 50% of Stx1A suffices for fusion and thereby we could not detect any effect in GSIS after siRNA silencing. Regardless, the absence of effect in the knock-down experiments in INS1-832/13 cells does not exclude that reduced expression of *STX1A* has a role in decreasing the insulin release in human islets.

The fact that we could not find any correlations in human islets between the expression levels and insulin secretion at 1 mM glucose (basal insulin secretion), when the Ca^{2+} influx through voltage-dependent Ca^{2+} channels are inhibited, suggests that the expression of exocytotic genes is mainly important for Ca^{2+}-dependent exocytosis evoked by an increase in glucose concentration. This is in line with our findings that the expression levels of several members of the synaptotagmin family, thought to act as the main Ca^{2+}-sensors of exocytosis, are down-regulated in T2D donors.

This is the first study demonstrating that expression of *SYT4, 7, 11* and *13* in human islets correlates positively with GSIS and is reduced in islets from T2D donors. In addition, both SYT4 and SYT11 protein levels were significantly reduced. In this context it is of interest that SYT11 has not previously been shown to be expressed in pancreatic islets. Syt7 have earlier been demonstrated to be present in β-cells in rodents (Gut et al., 2001) and depletion of Syt7 leads to reduced first phase insulin secretion (Gustavsson et al., 2008,Li, Wang, Xu et al., 2007). This agrees with our results from the human islets (Table 2), but we could not observe any significant reduction of secretion in INS1-832/13 after siRNA silencing of Syt7.

Syt4 is the most conserved member of the synaptotagmin family and several functions of Syt4 have been proposed in neurons. These include maturation of granules (Ahras, Otto and Tooze, 2006) and both stimulatory and inhibitory effect on exocytosis (Mori and Fukuda, 2011). The protein is present in insulin secreting cells (Gut et al., 2001), but no study have presented any effect on insulin exocytosis. Here we demonstrate that SYT4 gene expression is reduced in islets from T2D donors (Figs. 1 and 2) with a concomitant decrease in SYT4 protein level (Fig. 3c and d). Moreover, siRNA silencing of *Syt4* significantly reduce GSIS in insulin-secreting cells (Fig 5b). Thus, it can be suggested that SYT4 take part in the regulation of insulin secretion, by a yet not fully elucidated mechanism.

We detect a reduced gene expression of *SYT13* in islets from T2D donors which is in line with reduced *Syt13* gene expression in the diabetic Zucker rat (Parton et al., 2006). In addition, SYT13 gene expression is positively correlated to GSIS (Fig. 4b, Tables 2 and S2) and silencing of Syt13 reduced insulin secretion in INS1-832/13 cells (Fig. 5b). The protein level of SYT13 in human islets was not significantly different between the two groups why the observed down-

regulation of *SYT13* gene expression might be a false-positive finding. However, the immunoblot analysis was performed on islets from few individuals, due to limited amount of material. Moreover, the insulin secretion measurements clearly demonstrate a role for SYT13 in insulin secretion (Figs. 4b and 5b).

The positive correlation between gene expression levels and GSIS in human islets for *STX1A*, *SYT4, SYT7, SYT11*, *SYT13*, *SNAP25* and *STXBP1* suggest a possible role of these genes in maintaining glucose homeostasis. This fits with the concomitant negative correlation to HbA1c. It is uncertain if the reduced expression is causing the increase in blood glucose or if the change in expression is a result of the hyperglycemia. In our hands, siRNA silencing of exocytotic genes was followed by reduced insulin secretion, indicating that reduced gene expression may contribute to increases in blood glucose. It can be questioned if the impaired insulin secretion seen in the islets from T2D donors can be due to the reduced insulin content in these islets. However, expression of most exocytotic genes still correlate either negatively to HbA1c and/or positively to GSIS (Table S2), when correlation analysis was performed in non-diabetics only where insulin content was not reduced.

According to our cut-off points we found that two SNPs: rs12524194 and rs1339226 in the gene *RIMS1* were nominally associated with 2-h glucose and insulinogenic index in non-diabetic individuals of DGI. However, we were unable to replicate this finding in a population-based replication cohort from Botnia, Finland. Nevertheless, we found that the G-allele of rs12524194 was associated with reduced expression of *RIMS1* in human islets (Fig. 6). Interestingly, carriers of the G-allele displayed reduced insulin secretion (Table S4). Given the rather extensive SNP analysis of the exocytotic genes performed, we are tempted to conclude that single polymorphisms of these genes seem not to be a primary cause of impaired insulin secretion. Yet, it is possible that if we were to investigate further upstream or downstream sections or used larger cohorts, additional SNP associations could be detected. The down-regulation of the exocytotic genes may instead be attributed to epigenetic changes. Indeed, epigenetic factors including DNA methylation and histone modifications have been associated with decreased gene expression related to reduced insulin secretion in human islets (Kaneko, Ueki, Takahashi et al., 2010,Sandovici, Smith, Nitert et al., 2011,Yang, Dayeh, Kirkpatrick et al., 2011). Furthermore, changes in gene expression can also be ascribed to the action of microRNAs (miRNAs); small non-coding RNAs that infers translational regulation of specific target proteins (Guay, Roggli, Nesca et al., 2011). We have recently detected an up-regulation of rno-miR-335 in the diabetic GK-rat model (Esguerra, Bolmeson, Cilio et al., 2011) and validated its target protein Stxbp1. Furthermore, the same miRNA putatively targets Snap25. Finally, it has been demonstrated that β-cell specific knock-out of PI3K in mice leads to reduced expression of Snap25, Stx1A and Vamp2 via impaired PI3K/Akt pathway and the transcription factor FoxO1 (Kaneko et al., 2010). In this context, it is interesting that we found that the expression of *SNAP25* and *STXBP1* in human islets positively correlated to GSIS although we could not detect any SNPs that associated with reduced expression of these genes (data not shown).

There is increasing evidence that impairment of the exocytotic machinery is one of several contributing factors in the pathogenesis of T2D. This is further strengthened by this study, where expression of some of the key players involved in this process are demonstrated to be altered in islets from T2D donors, and where reduced expression has been shown to translate into decreased insulin secretion. Additional studies are needed to further evaluate the role of e.g. SYT4 in human islets insulin secretion and the relationship between expression of exocytotic genes and development of T2D.

Acknowledgements

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TABLES

Table 1 Characteristics of human pancreatic donors.

	Non-diabetic donors	T2D donors	*p*-value
n (male/female)	55 (29/26)	9(5/4)	
Age (years)	56.7 ± 9.8	57.0 ± 13.1	0.99
BMI (kg/m^2)	25.9 ± 3.6	28.5 ± 4.7	0.13
HbA$_1$c (%)	5.7 ± 0.8	7.3 ± 1.2	**0.00010**
HbA$_1$c (mmol/mol)	38.8 ± 8.7	56.2 ± 12.9	**0.00010**
Basal insulin secretion (ng/islet/h)	0.22 (0.39)	0.22 (0.17)	0.87
Glucose-stimulated insulin secretion (ng/islet/h)	1.06 (0.86)	0.54 (0.56)	**0.025**
Insulin content (ng/ml)	18650 (16881)	9370 (9244)	**0.043**

Data are expressed as mean ± SD or median (IQR). *p*-values calculated with Mann-Whitney U-test or Independent samples median test.

Table 2. Spearman correlations (ρ) between expression of exocytotic genes and HbA1c, GSIS and BIS in human donors.

Gene	HbA1c (n=52)		GSIS (n=48)		BIS (n=48)	
	ρ	p-value	ρ	p-value	ρ	p-value
RIMS1	0.189	0.180	-0.006	0.968	0.59	0.689
RIMS2	**-0.393**	**0.004**	0.265	0.069	-0.090	0.541
SNAP25	**-0.327**	**0.018**	**0.295**	**0.042**	0.092	0.535
STX1A	**-0.386**	**0.005**	**0.409**	**0.004**	0.198	0.178
STXBP1	**-0.371**	**0.007**	**0.300**	**0.038**	0.025	0.868
STXBP2	**0.379**	**0.006**	-0.238	0.104	0.079	0.595
STXBP3	0.087	0.541	0.118	0.423	0.193	0.189
SYT1	-0.81	0.567	0.264	0.069	0.190	0.196
SYT2	-0.123	0.386	-0.075	0.614	-0.124	0.403
SYT3	0.010	0.944	-0.151	0.307	0.054	0.713
SYT4	**-0.377**	**0.006**	**0.393**	**0.006**	0.079	0.591
SYT5	-0.198	0.160	0.269	0.064	-0.008	0.959
SYT6	0.020	0.890	-0.103	0.487	-0.051	0.729
SYT7	**-0.318**	**0.022**	**0.298**	**0.039**	0.047	0.753
SYT8	-0.159	0.259	0.028	0.851	0.019	0.896
SYT9	-0.097	0.496	-0.049	0.742	-0.147	0.318
SYT11	**-0.271**	**0.052**	**0.355**	**0.013**	0.120	0.418
SYT13	**-0.458**	**0.001**	**0.440**	**0.002**	0.130	0.379
VAMP2	**-0.287**	**0.039**	0.250	0.087	-0.066	0.656
UNC13A	-0.269	0.054	0.266	0.068	0.034	0.818
UNC13B	-0.187	0.185	-0.095	0.522	-0.023	0.875
UNC13C	0.032	0.823	-0.128	0.385	-0.083	0.575
UNC13D	-0.014	0.919	-0.262	0.072	-0.091	0.536

Data were obtained from non-diabetic and T2D donors and are presented by the Spearman correlation factor (ρ) and p-value of the association. Significant correlations ($p < 0.05$) are indicated in bold. GSIS-Glucose Stimulated Insulin Secretion; BIS- Basal Insulin Secretion

Figure 1. Microarray expression patterns of exocytotic genes from pancreatic islets of non-diabetic (n=55) and T2D (n=9) donors. Bars represent mean values ± s.d. of mRNA expression (a.u.). *p≤0.05; **p≤0.01. p-values are not corrected for multiple testing.

Figure 2. Gene expression of *STX1A*, *SYT4* and *SYT7* in human pancreatic islets of non-diabetic (n=43) and T2D (n=7) donors measured by qRT-PCR. Bars represent mean values ± s.d. of relative mRNA expression. p-values are based on Mann-Whitney U-test (1-sided) and not corrected for multiple testing. *p<0.05

Figure 3. Proteins levels of STX1A, SYT4 and SYT11 in human pancreatic islets. **a**, Immunostainig of STX1A in islet from non-diabetic (ND; top) and T2D (bottom) donor. **b**, Summary of STX1A mean intensity in β-cells. It was ascertained that all settings were the same between all images taken. Data are presented as mean±s.e.m in ND (n=37 cells from N=2 donors) and T2D donors (n=14 cells from N=1 donor). *p<0.05. **c**, Representative western blots demonstrating protein expression of SYT4, SYT7 SYT11 and SYT13 in human pancreatic islets from one non-diabetic (ND) and one T2D donor. PPIB (~ 20-25 kDa) and rat brain (RB) homogenate were used as loading control and control for each antibody, respectively. Notice that the antibody against SYT 11 only was immunoreactive to human samples. **d**, Histogram representing protein expression relative to PPIB from experiments in c. Protein expression was normalized to mean-value in islets from ND donors. Data used for analyses is based on 2-5 blots per donor and protein investigated. Quantified data are presented as mean±s.e.m in islets from ND (N=2 donors) and T2D donors (N=3 donors). *p<0.05 using Mann-Whitney U-test.

Figure 4. Gene expression of *STX1A* and *SYT13* are correlated with HbA1c and glucose stimulated insulin secretion (GSIS). **a**, Spearman correlation between *STX1A* gene expression and GSIS performed on data from islets derived from non-diabetics donors alone (black line; N=41 donors) or on islets derived from non-diabetic and T2D donors (red dotted line; N= 48 donors). Data obtained on islets from T2D individuals are marked with red. **b**, As in (a) but the correlation between *SYT13* gene expression and GSIS was investigated. **c,** As in (a) but the gene expression of *STX1A* was correlated to HbA1c. N=52 donors altogether and N=44 non-diabetic donors. **d,** As in (c) but the correlation between *SYT13* gene expression and HbA1c was investigated. Spearman correlation coefficients and p-values derived from all donors or non-diabetic donors alone are presented in Tables 2 and S2, respectively.

Figure 5. Effects of siRNA against Stx1a, Syt4, Syt7, Syt11 and Syt13 on GSIS in INS1-832/13 cells. **a**, Expression of genes following transfection of INS1-832/13 cells with scramble siRNA and siRNA against specific genes as indicated. **b**, GSIS after siRNA treatment, Data is normalized to insulin secretion at 16.7 mM glucose from cells transfection with scramble siRNA. In cells transfected with scramble siRNA insulin release at 2.8 mM and 16.7 mM glucose was 9.8±1.3 ng/mg protein/h (n=5) and 70.0±6.2 ng/mg protein/h (n=5; p<0.001 *vs* 2.8 mM),

respectively. Data is presented as mean±sem of n=5 experiments in each group. ***p<0.001 and **p<0.01 *vs* scramble using Students t-test corrected by the Holm-Bonferroni-method.

Figure 6. Expression of *RIMS1* (a.u.) in islets with different genotypes of rs12524194. *p<0.05 *vs* the expression of *RIMS1* in AA carriers.