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Published in:
Endocrinology

DOI:
[10.1210/en.2011-1702](https://doi.org/10.1210/en.2011-1702)

2012

[Link to publication](#)

Citation for published version (APA):

Wendt, A., Speidel, D., Danielsson, A., Esguerra, J. L. S., Bogen, I. L., Walaas, S. I., Salehi, S. A., & Eliasson, L. (2012). Synapsins I and II Are Not Required for Insulin Secretion from Mouse Pancreatic beta-cells. *Endocrinology*, 153(5), 2112-2119. <https://doi.org/10.1210/en.2011-1702>

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Synapsins I and II Are Not Required for Insulin Secretion from Mouse Pancreatic β -cells

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Synapsins are a family of phosphoproteins that modulate the release of neurotransmitters from synaptic vesicles. The release of insulin from pancreatic β -cells has also been suggested to be regulated by synapsins. In this study, we have utilized a knock out mouse model with general disruptions of the *synapsin I* and *II* genes [synapsin double knockout (DKO)]. Stimulation with 20 mM glucose increased insulin secretion 9-fold in both wild-type (WT) and synapsin DKO islets, whereas secretion in the presence of 70 mM K^+ and 1 mM glucose was significantly enhanced in the synapsin DKO mice compared to WT. Exocytosis in single β -cells was investigated using patch clamp. The exocytotic response, measured by capacitance measurements and elicited by a depolarization protocol designed to visualize exocytosis of vesicles from the readily releasable pool and from the reserve pool, was of the same size in synapsin DKO and WT β -cells. The increase in membrane capacitance corresponding to readily releasable pool was approximately 50fF in both genotypes. We next investigated the voltage-dependent Ca^{2+} influx. In both WT and synapsin DKO β -cells the Ca^{2+} current peaked at 0 mV and measured peak current (I_p) and net charge (Q) were of similar magnitude. Finally, ultrastructural data showed no variation in total number of granules (N_t) or number of docked granules (N_d) between the β -cells from synapsin DKO mice and WT control. We conclude that neither synapsin I nor synapsin II are directly involved in the regulation of glucose-stimulated insulin secretion and Ca^{2+} -dependent exocytosis in mouse pancreatic β -cells. (*Endocrinology* 153: 2112–2119, 2012)

Type-2 diabetes is caused by a combination of peripheral insulin resistance and defective insulin secretion, and it has been shown that impaired insulin secretion precedes the onset of the disease (1). Insulin is secreted in a Ca^{2+} -dependent manner from pancreatic β -cells in response to elevated blood glucose levels. Within the β -cell, insulin is packed in large-dense core vesicles (LDCV) that are believed to be functionally divided into the readily releasable pool (RRP) of vesicles that can undergo exocytosis immediately upon stimulation and the reserve pool (RP) that have to undergo further modifications before

release. Although endocrine β -cells have been shown to be of different developmental origin than neurons, the two cell types share many similarities regarding their exocytotic machinery including the expression of synapsins (2–4).

Synapsins are a family of phosphoproteins, mostly described in association with synaptic vesicles in neurons, but synapsins have also been implicated in the release of catecholamines (5) as well as insulin (3, 6, 7) from LDCVs. In the original model (8) synapsins were believed to tether the vesicles in RP to each other and to the neighboring

ISSN Print 0013-7227 ISSN Online 1945-7170

Printed in U.S.A.

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doi: 10.1210/en.2011-1702 Received August 31, 2011. Accepted January 19, 2012.

First Published Online February 14, 2012

Abbreviations: DKO, Double knockout; LDCV, large-dense core vesicles; qPCR, quantitative PCR; RP, reserve pool; RRP, readily releasable pool; WT, wild type.

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actin network in the synapse, thereby preventing them from reaching the plasma membrane. Upon activation, the synapsins are phosphorylated, which allows them to dissociate from the vesicles. This allows the vesicles to freely move to and fuse with the plasma membrane. Later, synapsins have been suggested to play a role in processes such as docking and endocytosis (for a review see Ref. 9). Three mammalian genes for synapsin (*synapsin I–III*) have been characterized (10, 11). Of these, *synapsin I* has been shown to be expressed in rat islets as well as in the insulin-secreting cell lines MIN-6 (3) and β TC-3 (12). It has been suggested that phosphorylation of synapsin I, by ERK 1/2 (6) or Ca^{2+} /CaM-dependent kinase II (7, 13), may play a role in insulin secretion although contradictory results has also been obtained (12). The exact role of synapsins in pancreatic β -cells therefore remains unclear.

Previous studies have not aimed at showing a direct effect of synapsins on insulin secretion. In this study, we have used a knock out mouse model where the *synapsin I* as well as the *synapsin II* gene have been inactivated [Synapsin double knockout (DKO)]. Islets and/or single β -cells from these mice were investigated with respect to insulin release, depolarization-evoked exocytosis, and morphological changes in ultrastructure. We found that knocking out the *synapsin I* and *II* genes had no effect on either the distribution or the release of insulin containing large dense core vesicles but appeared to enhance the secretion of insulin vesicles during prolonged incubation with 70 mM K^+ .

Materials and Methods

Animals

Synapsin DKO mice, a gift of Professor Greengard (The Rockefeller University, New York, NY), were obtained by homologous recombination as previously described (14–16) and compared with genetically matched wild-type (WT) mice (C57BL/6). The mice were maintained on a 12-h light, 12-h dark cycle with free access to food and water. All experimental procedures involving animals were approved by the ethical committee in Lund.

Islet isolation and islet cell preparation

Pancreatic islets of Langerhans were isolated from Synapsin DKO and WT mice (C57BL6 background) as well as C57BL6 and NMRI mice [for RT-quantitative PCR (qPCR)]. The mice were killed by cervical dislocation, and pancreatic islets were isolated by collagenase digestion as described elsewhere (17). Single cells were obtained by trituration of islets in a Ca^{2+} -free solution. The cells were cultured in RPMI-1640 media (5 mM glucose) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin and kept at 37°C in a humidified incubator containing 5% CO_2 and 95% air.

RNA isolation and RT-qPCR

RNA was extracted from 100–300 pancreatic islets isolated from three individual NMRI mice (no pooling) or two C57BL6 mice using RNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. The extracted RNA was quantified using Nanodrop 1000 (Thermo Fisher Scientific, Inc., Waltham, MA), and the quality was evaluated using spectrophotometric ratios and electropherogram profiles (Experion systems, Bio-Rad Laboratories, Hercules, CA). For reverse transcription, 100 ng of total RNA were used in a 20- μl reaction using Applied Biosystems' High Capacity cDNA kit (Applied Biosystems, Foster City, CA). After RT the cDNA was diluted four times and 4 μl were used in the qPCR step using gene-specific Taqman expression assays (Applied Biosystems). All assays were performed in a 10 μl reaction volume in triplicate. The Taqman assays used were as follows *Synapsin I*; Mm 00449772_m1, *Synapsin II*; Mm 00449780_m1, *Synapsin III*; Mm 00489077_m1. As reference gene we used *Hprt I*; Mm 00446968_m1.

Immunocytochemistry

Islets from NMRI mice were dissociated into single cells and seeded on glass cover slips coated with poly-L-lysine (Sigma, Karlstad, Sweden). When the cells had firmly attached, they were treated essentially as described elsewhere (18). The cells were coincubated with the primary antibodies; guinea pig α -insulin; dilution 1:1000 (to detect β -cells; LINCO Research, Inc., St. Charles, MO) and the synapsin I- and II-specific antibody α -synapsin; dilution 1:100 (SySy, Goettingen, Germany). As secondary antibodies Cy5 donkey α -guinea pig; dilution 1:200 and DyLight 488 donkey α -rabbit; dilution 1:200 (both from Jackson ImmunoResearch Europe Ltd, Suffolk, UK) were used. The labeled cells were viewed using a LSM 510 confocal microscope (Carl Zeiss, Jena, Germany). Unspecific binding of the secondary antibodies was excluded in control experiments in which the primary antibodies were omitted.

Hormone release measurements

Insulin and glucagon release was examined using RIA as described elsewhere (19, 20). Briefly, batches of 12 islets were pre-incubated for 30 min in Krebs-Ringer buffer supplemented with 1 mM glucose followed by 1 h incubation in Krebs-Ringer buffer containing 1, 8, or 20 mM glucose and 70 mM KCl (K^+) or 10 μM forskolin as indicated. When KCl was included in the solution, NaCl was equally reduced to keep the osmolality of the buffer.

Electrophysiology

Patch pipettes were pulled from borosilicate glass (tip resistance, 3–6 M Ω when filled with pipette solution). The electrophysiological measurements were performed using the standard whole-cell configuration. The measurements were conducted using an EPC-9 or EPC-10 amplifier and the software Pulse (Heka Elektronik, Lambrecht, Germany; version 8;31) β -Cells were identified based on their size and on the inactivation properties of the voltage-gated Na^+ -channels (21).

The standard extracellular solution consisted of (in millimolar concentration) 118 NaCl, 20 TEACl, 5.6 KCl, 2.6 CaCl_2 , 1.2 MgCl_2 , 5 HEPES, and 5 glucose (pH 7.4 with NaOH). The intracellular solution consisted of (in millimolar concentration)

125 Cs-Glut, 10 NaCl, 10 CsCl, 1 MgCl₂, 0.05 EGTA, 3 Mg-ATP, 10 HEPES, and 0.1 cAMP (pH 7.15 using CsCl).

Transmission electron microscopy

The whole pancreas was dissected out from Synapsin DKO and WT mice, fixed in 4% paraformaldehyde, and stored at 4°C. The pancreases were sliced with a scalpel to smaller sections (1–2 mm), which were incubated in 2.5% glutaraldehyde overnight and treated with 1% osmium tetroxide for 2 h, dehydrated, and then embedded in AGAR 100 (Oxford Instruments Nordiska AB, Sweden). Finally they were cut in 70- to 90-nm sections, put on Cu-grids, and contrasted with uranyl acetate and lead citrate. The samples were examined in a JEM 1230 electron microscope (JEOL-USA, Inc., Peabody, MA), and the micrographs were analyzed with respect to LDCV density and docked LDCV density as previously described (22). The diameter of individual vesicles was determined using Scion Image (NIH free-ware). The granule volume density (N_v) and surface density (N_s) were calculated using in-house software programmed in MatLab (version 7x).

Statistical analysis

Data are given as mean \pm SEM. Statistical significance was evaluated using Student's *t* test.

Results

Synapsins are present in mouse islets

It has previously been reported that synapsin I is expressed in pancreatic β -cell lines and in primary rat islets (3, 12, 13). To study the expression of all three *synapsin* genes in mouse islets we performed qPCR on whole islets from NMRI and C57Bl6 mice. We found that *synapsin I* is the most dominant of the three isoforms in the islets, followed by *synapsin II*, whereas *synapsin III* was present in relatively low concentrations (Fig. 1, A and B). Synapsin I and/or II was also detected at protein level in mouse β -cells using immunocytochemistry (Fig. 1C).

Hormone secretion measurements from synapsin DKO islets

To investigate the role of synapsin I and II on insulin secretion, we compared hormone release from islets from synapsin DKO and WT mice (15). The islets were incubated in 1, 8, and 20 mM glucose (Fig 2A). In WT islets, insulin secretion was increased approximately 3.4-fold at 8 mM ($P < 0.001$ vs. 1 mM glucose) and about 9.2-fold at 20 mM glucose ($P < 0.001$ vs. 1 mM glucose). Similar responses were observed in the synapsin DKO. Interestingly, stimulation by 70 mM K⁺ at 1 mM glucose augmented insulin secretion to a higher extent in the synapsin DKO than the WT islets (4.4-fold vs. 3.3-fold; $P < 0.001$). Synapsins are phosphoproteins with their activity being regulated by kinases. The cAMP-dependent Protein kinase

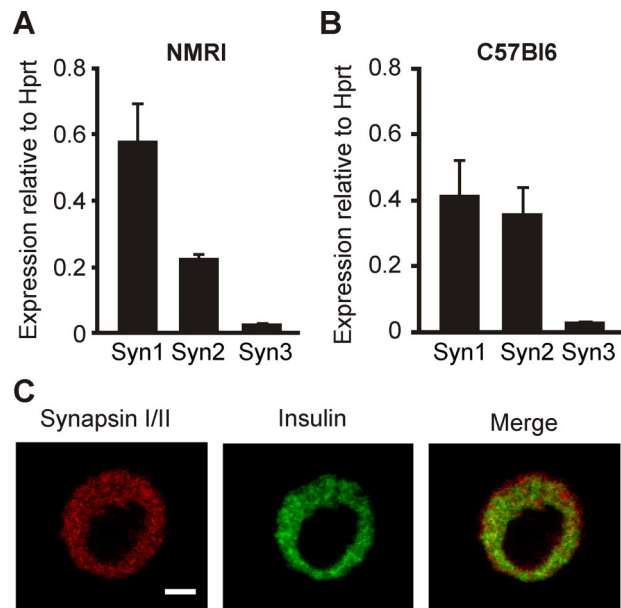


FIG. 1. Relative quantification of synapsin isoforms in isolated mouse islets. mRNA levels of the three known synapsin isoforms were determined relative to the reference gene *Hprt* using gene-specific Taqman assays in NMRI (A) and C57Bl6 (B) mouse islets. Data are expressed as mean \pm SEM of three mice and three technical replicates of each mouse. C, Confocal image of a single mouse β -cell expressing insulin (green) and synapsin I and/or II (red). The image is a representative of 13 investigated cells. Scale bar, 2 μ m. Syn, Synapsin.

A is a kinase that is important for insulin secretion (23). We therefore investigated whether forskolin, which activates adenylate cyclase and thereby increases the intracellular concentration of cAMP (24), was less efficient in enhancing insulin secretion from synapsin DKO islets. Although there was a tendency toward decreased forskolin effect in the synapsin DKO, there was no significant difference between synapsin DKO and WT islets (1.7 ± 0.3 -fold vs. 2.2 ± 0.2 -fold increase compared with 20 mM glucose alone).

Furthermore, glucagon released from α -cells and somatostatin secreted from δ -cells in the islets of Langerhans are paracrine regulators of insulin secretion (25), and the observed insulin responses might be influenced by effects of the deletion of synapsin I and II on glucagon and/or somatostatin secretion. When investigated, there were no alterations in the release of either of these hormones in the conditions tested (Fig 2, B and C).

Depolarization-induced exocytosis in β -cells lacking synapsin I and II

Next we used the standard whole-cell configuration of the patch clamp technique to examine exocytosis from single β -cells. Exocytosis was elicited by a train of depolarizations consisting of five short 100-msec depolarizations to 0 mV followed by nine 500-msec depolarizations to 0 mV (Fig 3, A–C). This protocol was designed to vi-

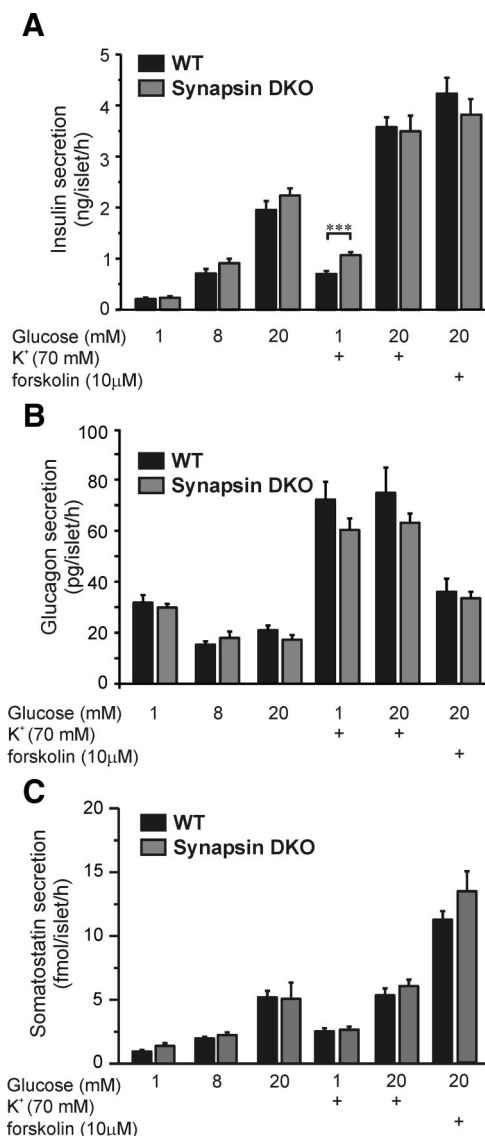


FIG. 2. Hormone secretion measurements from islets depleted of *synapsin I* and *II*. Insulin secretion (A) glucagon secretion (B), and somatostatin secretion (C) from islets incubated in low (1 mM), medium (8 mM), and high (20 mM) concentrations of glucose in the presence or absence of 70 mM K⁺ or 10 μM forskolin as indicated in the figure. Black bar represents the WT islets whereas gray bars represent the synapsin DKO islets. Data are expressed as mean ± SEM from 12 experiments, ***, $P < 0.001$.

sualize exocytosis of vesicles from RRP (membrane capacitance increase evoked by the first five short depolarizations combined with the first 500-msec depolarization) and from RP (membrane capacitance increase evoked by the following eight depolarizations). Analysis of the data revealed that the increase in membrane capacitance corresponding to RRP was not different between β -cells from synapsin DKO and WT mice [43 ± 7 femtofarads (fF); $n = 16$ vs. 57 ± 23 fF; $n = 13$]. Similarly, no significant difference was seen in the response to the last eight depolarizations (112 ± 16 fF vs. 92 ± 21 fF) or in the total response to the train (166 ± 29 fF vs. 163 ± 43 fF.). These

data are summarized in Fig. 3D. It was ascertained that the average β -cell size did not differ between the synapsin DKO and WT cells in these experiments (6.5 ± 0.3 fF vs. 6.8 ± 0.3 fF).

Characterization of the voltage-dependent Ca²⁺ influx in *synapsin I/II*-deficient β -cells

Exocytosis is closely linked to Ca²⁺ influx through voltage-gated Ca²⁺ channels (26). To investigate whether synapsins influence Ca²⁺ currents, we investigated the peak current-voltage (I_p V) and charge-voltage (QV) relationships in single β -cells from synapsin DKO and WT mice. We did this by applying depolarizations from -50 mV to $+20$ mV to the β -cells. The corresponding peak current (I_p) and charge (Q) passing through the channels was then measured. The maximal Ca²⁺ current is evoked by a depolarization to 0 mV in both synapsin DKO and WT β -cells (Fig. 4C). The peak current was equally large in synapsin DKO and WT cells (71 ± 38 pA; $n = 11$ vs. 78 ± 22 pA; $n = 16$). Similarly there was no difference in I_p or Q at any voltage tested (Fig. 4, C and D).

Ultrastructural analysis of LDCV in β -cells from synapsin DKO mice

Finally, we investigated whether deletion of *synapsin I* and *II* had any effect on the distribution of insulin-containing vesicles in the β -cells. Figure 5A-B shows electron micrographs from a β -cell situated in an intact islet of a WT and a synapsin DKO pancreas, respectively. Analysis of the samples revealed no significant difference between the synapsin DKO and the WT β -cells with respect to the total amount of LDCV measured as LDCV volume density [N_V ; 9.1 ± 0.5 vesicles/ μm^3 ($n = 21$ cells) vs. 9.1 ± 0.4 vesicles/ μm^3 ($n = 19$ cells)]. Neither was the surface density (N_S) used to estimate the number of docked LDCVs altered between the genotypes [0.8 ± 0.1 vesicles/ μm^2 ($n = 21$ cells) vs. 0.7 ± 0.1 vesicles/ μm^2 ($n = 19$ cells)]; Fig. 5C]. To analyze for differences in localization of LDCVs, we investigated the distribution of the vesicles within specific distances from the plasma membrane. These results are summarized in a histogram in Fig. 5D. It is evident that there is no difference in vesicle distribution between synapsin DKO and WT β -cells.

Discussion

Insulin released from pancreatic β -cells plays a vital role in the homeostatic control of blood glucose. Because of this, the mechanisms behind insulin release have drawn the attention of many researchers over the years. The exocytotic machinery in β -cells resembles, in many aspects, processes

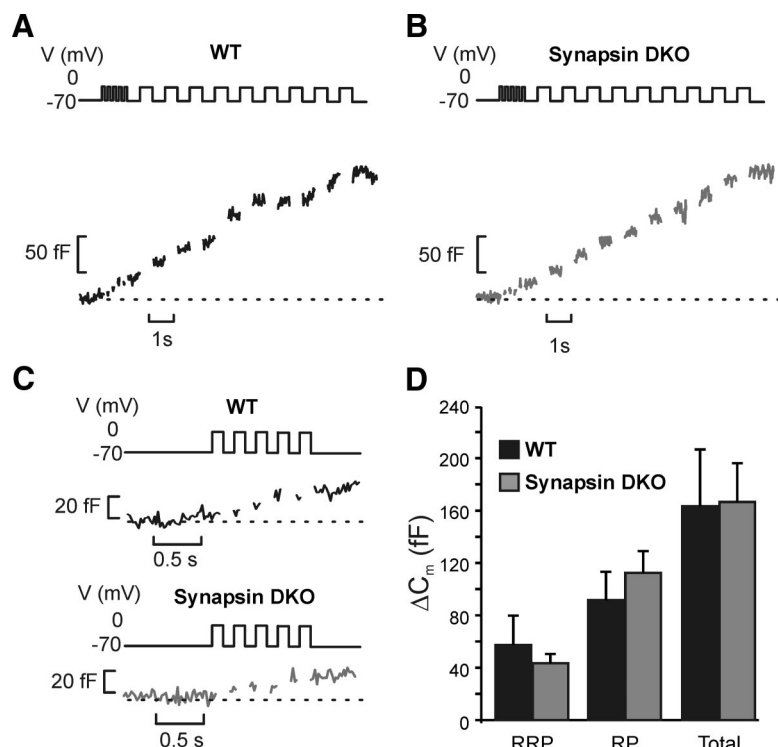


FIG. 3. Depolarization-induced exocytosis in β -cells lacking synapsin I and II. Example traces of the capacitance increase in single β -cells from WT (A) and synapsin DKO (B) mice evoked by a train of five short 100-msec depolarizations to 0 mV followed by nine longer 500-msec depolarizations to 0 mV. C, The capacitance responses in panels A and B during the five short depolarizations displayed at an expanded time scale. D, Summary of the data in which RRP represents the summed capacitance increase evoked by the first five short pulses and the first 500-msec pulse. RP represents the capacitance change stimulated by the eight remaining depolarizations. Total is the total capacitance increase initiated by all depolarizations of the train. WT is shown in black and the synapsin DKO is shown in gray bars. Data are expressed as mean \pm SEM from 13–16 cells. s, Seconds.

in neurons (27). Proteins that have been denoted an exocytotic function in neurons such as synapsins are therefore interesting to examine in the β -cell. The suggestion that synapsin I is involved in insulin secretion is based on the finding that the protein is phosphorylated by the kinases calmodulin kinase II and ERK 1/2, both of which are indicated to play a role in insulin secretion (6, 7). However, a direct effect of synapsin I in insulin secretion or insulin vesicle exocytosis was never examined in these studies, but rather the authors relied on circumstantial evidence. In addition, these studies were mainly performed on insulin-secreting cell lines such as MIN-6 rather than primary β -cells. Using a mouse knockout model depleted of both *synapsin I* and *II*, we here show that neither of the two synapsin forms are important for release of insulin. This is the first attempt to show a direct link between synapsins and insulin secretion.

We examined the release of insulin both by biochemical hormone measurements in intact islets by RIA and exocytosis of insulin vesicles in single β -cells by electrophys-

iological measurements. Hormone secretion from batch-incubated islets showed a slight increase in insulin secretion from the synapsin DKO islets in the presence of low glucose and high K^+ compared with WT (Fig. 2A). This condition was designed to investigate the first phase of insulin secretion, which is believed to correspond to exocytosis of vesicle belonging to the RRP (28). In agreement, it has been reported that exocytosis of RRP of LDCV in adrenal chromaffin cells, as well as release of neurotransmitter vesicles in brain and neuromuscular junction, can be negatively regulated by synapsins (5, 29, 30). However, our secretion data could not be verified at the single-cell level, when exocytosis was measured as cell membrane capacitance increase in response to membrane depolarizations (Fig. 3). These data excluded the possibility that docking and priming of insulin vesicles depend on synapsins I/II. Furthermore, the capacitance data correlated with the ultrastructural analysis, which showed no difference in the amount of docked vesicles between synapsin DKO and WT β -cells (Fig. 5C). The discrepancy between the results from the secretion data using high K^+ and the capacitance measurements could be due to paracrine effects, because secretion is measured on whole islets and exocytosis in single cells. However, neither glucagon nor somatostatin secretion

is altered in the synapsin DKO mouse (Fig. 2, B and C). We can only speculate regarding other mechanisms. Possibly, high K^+ for 1-h and capacitance measurements evokes different increases in intracellular Ca^{2+} concentration and/or spreading of Ca^{2+} in the cell. It has been reported that low-frequency stimulation of hippocampal slices from the synapsin DKO at 2 mM extracellular Ca^{2+} result in enhanced responses, whereas the DKO hippocampal neurons have a decreased response at other frequencies and/or Ca^{2+} concentration (29). Nevertheless, we believe that the response to high K^+ is an artificial effect that is not occurring under physiological conditions. Therefore, we suggest that the presence of synapsin I and II has little, if any, effect on the first phase of insulin release. In excitatory synapses, the synapsins have been reported to play a role in maintaining vesicles in the reserve pool (31). A similar effect could not be seen in insulin-secreting β -cells judging from our electrophysiological measurements and ultrastructural data. In conclusion, these data indicate that insulin release from pancreatic β -cells are independent of

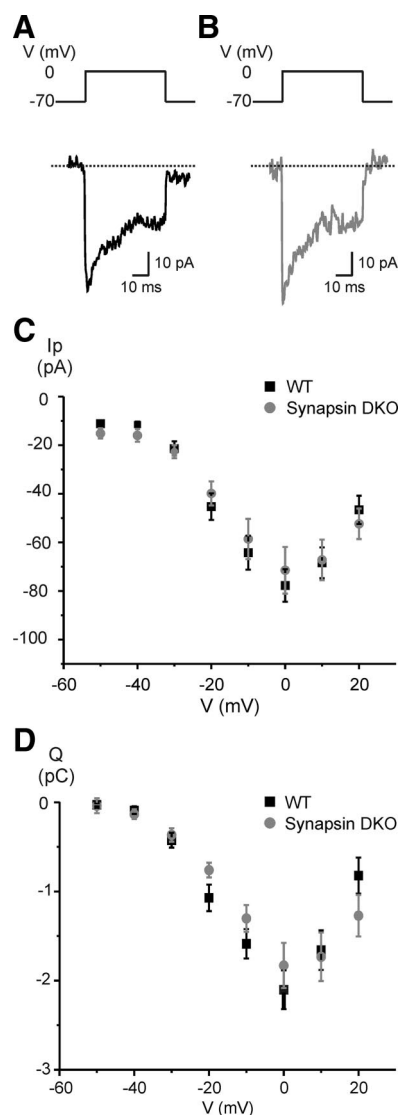


FIG. 4. Electrophysiological investigation of the voltage-dependent Ca^{2+} current in synapsin DKO β -cells. Example Ca^{2+} current traces measured on a single WT (A) and synapsin DKO (B) β -cell after depolarization to 0 mV. C, Peak current (I_p)-voltage (V) relationship and D) Charge (Q)-voltage(V) relationship recorded from synapsin DKO (gray circles) and WT (black squares) β -cells. Data are expressed as mean \pm SEM from 11–16 cells. ms, Milliseconds.

synapsin I and II, much like what has been reported for serotonin release in substantia nigra pars reticulata from a triple knock out mouse lacking all three synapsins (32).

We find no effects of synapsins on insulin release, but there is still a possibility that synapsins contribute to functions in the β -cell other than exocytosis. It has been reported, for instance, that synapsins are involved in endocytosis and recycling of putative synaptic vesicles in *Drosophila* motor boutons (33). Although endocytosis was not specifically investigated, our data indicate that it is not altered in the synapsin DKO mice. First, there were no empty vesicles close to the plasma membrane and no difference in the total amount of docked vesicles in the

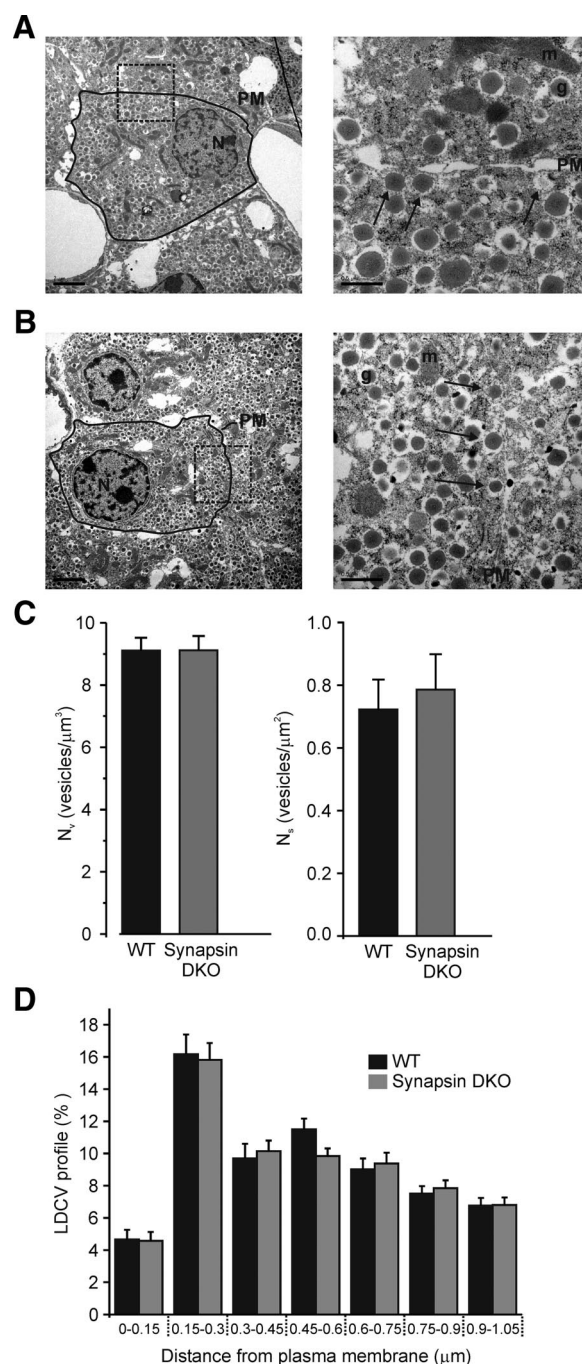


FIG. 5. Ultrastructural analysis of synapsin DKO β -cells. A, Electron micrograph of a synapsin WT β -cell within an islet (left); scale bar, 2 μm . The area within the square in the left image is highlighted to the right. Scale bar, 0.5 μm . Docked granules are indicated with arrows. PM, Plasma membrane; N, nucleus; g, granule; m, mitochondria. B, As in panel A but from a synapsin DKO mouse. C, Bar graphs showing (left) the total amount of LDCVs measured as volume density N_v (vesicles/ μm^3) and (right) the number of docked LDCVs measured as surface density N_s (vesicles/ μm^2). D, Histogram illustrating the percentage of LDCVs at different distances from the plasma membrane in WT (black bars) and synapsin DKO (gray bars) β -cells. Data are expressed as mean \pm SEM of 19–21 cells.

synapsin DKO mice (Fig. 5), suggesting proper vesicle recycling. In addition, if the vesicles were to be locked in their fusion state due to deregulated endocytosis, this would manifest itself as cells with a bigger surface area and therefore a bigger total cell capacitance. However, we could not detect any difference in cell size between the synapsin DKO and WT mice in our capacitance measurements.

Synapsins have been associated with neuronal disorders (34, 35), and the synapsin DKO mice exhibit an epileptic phenotype (2). This suggests that synapsins have a critical role in synapses. Other proteins involved in synaptic exocytosis have been demonstrated to be essential for insulin secretion; *e.g.* a mutation in *Snap-25* leads to a neurological disorder and reduced insulin secretion (36). Our data indicate that this is not true for synapsin I and II because the synapsin DKO does not have altered insulin exocytosis. Instead our data indicate that synapsin I and II are not needed for functional insulin release. Although synapsins are not involved in glucose-dependent secretion of insulin, we do not exclude that synapsins have other roles in the pancreatic β -cells, the identification of which remains interesting.

Acknowledgments

We thank Britt-Marie Nilsson at the Department of Clinical Sciences, Lund University and Rita Wallén at the Department of Functional Zoology, Lund University for excellent technical assistance. We thank Vini Nagaraj for sharing RNA from C57BLJ islets.

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This work was supported by Grants from the Swedish Research Council, the Novo Nordisk Foundation, The Swedish Diabetes Association, The Albert Pahlsson Foundation, Knut and Alice Wallenberg Foundation, Magnus Berwall Foundation, and O.E Edla Foundation. L.E is a senior researcher at the Swedish Research council.

Disclosure Summary: The authors have nothing to disclose.

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