Syntaxin 2 Acts as Inhibitory SNARE for
Insulin Granule Exocytosis

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ABSTRACT

Of the four syntaxins (Syns) specialized for exocytosis, syntaxin-2 is the least understood. Here, we employed syntaxin-2/epimorphin knockout (KO) mice to examine the role of syntaxin-2 in insulin secretory granule (SG) exocytosis. Unexpectedly, syntaxin-2 KO mice exhibited paradoxical superior glucose homeostasis resulting from an enhanced insulin secretion. This was confirmed in vitro by pancreatic islet perifusion showing an amplified biphasic glucose-stimulated insulin secretion (GSIS) arising from an increase in size of the readily-releasable pool of insulin SGs and enhanced SG pool refilling. The increase in insulin exocytosis was attributed mainly from an enhanced recruitment of the larger pool of newcomer SGs that undergoes no residence time on plasma membrane before fusion, and to lesser extent also the predocked SGs. Consistently, syntaxin-2 depletion resulted in stimulation-induced increase in abundance of exocytotic complexes we previous demonstrated to mediate the fusion of newcomer SGs (Syn-3/VAMP8/SNAP25/Munc18b) and predocked SGs (Syn-1A/VAMP2/SNAP25/Muncn18a). This work is first to show in mammals that syntaxin-2 could function as inhibitory SNARE protein, which when relieved, could promote exocytosis in pancreatic islet β-cells. Thus, syntaxin-2 may serve as a potential target to treat diabetes.
INTRODUCTION

Exocytosis of insulin SGs from pancreatic β-cells (1) mimics neurotransmitter release (2), with major components of the neuronal exocytotic fusion machinery, including the three SNARE proteins, Syn-1A, SNAP-25 and VAMP-2, and nSec/Munc18 (SM) protein Munc18a which acts to remodel and activate SNARE complex assembly. β-cells, unlike neurons, employ several modes of exocytosis from several SG pools to mediate a biphasic insulin secretory pattern that drives glucose uptake during a meal (3). Vesicle(v-)-target(t-) membrane SNARE (Syn-1A through -4, and SNAP25) and SM proteins (Munc18a,b,c), each constituting a family of isoforms, whereby cognate partners form distinct SM/SNARE complexes that underlie the molecular basis of each exocytotic event in β-cells (1-3). Neuronal Munc18a/Syn-1A/SNAP25/VAMP2 complex mediating exocytosis of predocked insulin SGs (and predocked synaptic vesicles) (4,5) accounts for first-phase of the biphasic GSIS. Newcomer insulin SGs are mobilized from the β-cell interior, taking minimal to no residence time at the PM before undergoing fusion (6,7). Newcomer SGs, accounting for almost all of second-phase GSIS and a major portion of first-phase GSIS, are mediated by another SM/SNARE complex, Munc18b/Syn-3/SNAP25/VAMP8 (8-10). The Munc18c/Syn-4 SM/SNARE complex exhibits redundant actions of mediating exocytosis of predocked and newcomer SGs (11).

This leaves Syn-2, which binds mainly Munc18b (also Munc18a but not Munc18c) (12), to be the only exocytotic syntaxin whose role in exocytosis is yet to be elucidated. Peculiarly, Syn-2, also called epimorphin, was better studied for its morphogenic role influencing intestinal growth (13-15) and mammary carcinogenesis (16). Syn-2’s role in exocytosis was reported in non-neuronal cells, including salivary (17) and pancreatic acini (18), sperm acrosome (19), lung alveoli (20) and platelets (21,22). An early report suggested that Syn-2 may not have a role in
insulin secretion (23).

Here, we determined Syn-2’s role in insulin exocytosis, employing the Syn-2-KO mouse (14; 15). Paradoxically, Syn-2-KO mice exhibited improved glucose homeostasis from increased biphasic GSIS attributed to accelerated recruitment and fusion of newcomer SGs and to lesser extent predocked SGs. Mechanistically, Syn-2 deletion resulted in increase in pro-exocytotic SM/SNARE complexes Syn-3/Munc18b/SNAP25/VAMP8 and Syn-1A/Munc18a/SNAP25/VAMP2. This suggests that Syn-2 likely acts as an inhibitory SNARE (24) in β-cell, which when abrogated relieves its competitive inhibition to enable formation of these pro-fusion SNARE complexes to promote insulin secretion.

RESEARCH DESIGN AND METHODS

Mouse Genetics and Islet Isolation

Syn-2-KO (Epimorphin<sup>−/−</sup>) mice were generated as described (14,15). All analyses in this paper on constitutive KO mice were performed with littermate control (WT mice). Animal procedures were performed in accordance with University of Toronto’s Animal Care Committee’s ethical guidelines. Islets were isolated by collagenase digestion and dispersed into single β-cells with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS containing 5 mM EDTA and 0.25 mg/ml trypsin. Islets, β-cells and INS-1, cultured in supplemented RPMI 1640 (Gibco) medium.

Western Blot, Subcellular Fractionation, Immunoprecipitation and In Vitro Binding Assays

Western blots of islet lysate samples were performed with the following antibodies: VAMP2 (1:2,000; Anson Lowe, Stanford University, Stanford, CA), VAMP8 (1:1000; CC Wang and WJ Hong, Institute of Molecular and Cell Biology Singapore), Syn-1A (1:1,000; Sigma, St. Louis, MO), from Synaptic Systems (Goettingen, Germany) include Syn-2 (1:1,200), Syn-3 (1:1000),
Syn-4 (1:1,200) and SNAP-23 (1:1,000); SNAP-25 (1:1,500; Sternberger Monoclonal, Covance, Princeton, NJ), Munc18a (1:1000; BD Transduction Laboratories, San Jose, CA), Munc18b (1:1,000; V. Olkkonen, Minerva Foundation Institute, Finland) and Munc18c (1:800; D. Thurmond, City of Hope, Duarte, CA).

Subcellular fractionation was performed as previously reported (25) with slight modifications (see Online Supplementary Methods). Briefly, INS-1 cells at 70–80% confluence were washed and harvested into homogenization buffer containing protease inhibitors. Cells were disrupted and divided into two tubes of homogenates. The first tube was subjected to centrifugation at 900g (10-min) to remove the nuclei-enriched fraction; postnuclear supernatants centrifuged at 5,500g (15-min), with subsequent supernatant centrifuged at 25,000g (20-min) to obtain the SG-enriched pellet; resulting supernatant then centrifuged at 100,000g (1-h) to obtain the cytosolic fraction. From the second tube, PM fractions were obtained by centrifugation at 900g, the resulting supernatant then centrifuged at 100,000g (1-h) to obtain a pellet. The pellet was mixed with equal volumes of Buffer A and Buffer B, this mixture overlaid with Buffer A, centrifuged at 113,000g (1-h) to obtain an interface containing the PM fraction, which was collected and pelleted (3,000g, 10-min) to obtain the enriched PM fraction. All fractions were assayed for soluble protein content.

Immunoprecipitation (IP) assays using Syn-1A, Syn-2 and Syn-3 antibodies were conducted on adrenal glands (5-6 glands per IP) from WT or Syn-2-KO mice or INS-1 cells transduced with Syn-2 si-RNA versus scramble control. 72-h post-transduced cells were washed with PBS and incubated with Krebs–Ringer HEPES buffer (30-min, 37°C) at 0.8 mM glucose to obtain uniform basal condition, then further incubated with 10 nM GLP-1 (30-min) followed by stimulation with 16.7 mM glucose (containing 10 nM GLP-1) for 30-min. Treated cells were
harvested and lysed by sonication in lysis buffer (25 mM HEPES, 100 mM KCl, 1.5% Triton X 100, protease inhibitors). The detergent extract (650-800 µg protein) was pre-cleared by incubation with 35 µl protein A agarose (50% slurry) for 1.5-h, then incubated with protein A agarose-crosslinked anti-Syn-1A, -2 or -3 Ab (2 µg) at 4°C for 1.5-h. Precipitated proteins were washed with IP buffer three times, separated on 12/15% SDS PAGE and identified with indicated antibodies.

In vitro binding assays were performed according to methods previously described (26). Briefly, GST (as control), GST-VAMP2 or GST-VAMP8 (250 pmol protein) were bound to glutathione agarose beads and incubated with lysate extracts of transfected HEK293 cells (4°C, 2-h). Then the beads were washed three times with lysis buffer and samples separated on 13% SDS-PAGE; Proteins of interest were identified with specific antibodies. Details in Online Supplementary Methods.

**Intraperitoneal Glucose Tolerance Test (IPGTT), Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test**

Intraperitoneal glucose (1g glucose/kg bodyweight) tolerance tests were performed after 15- to 16-h fasting at 10-12-weeks old male mice, and weighed. Blood glucose was measured at indicated times, and blood samples obtained at the same time point for insulin measurement. The plasma component of blood collected was assayed by insulin ELISA (Alpco Diagnostics, Salem, NH). OGTT was performed as reported previously (10) (see Online Supplementary Methods).

For insulin tolerance test, human biosynthetic insulin (Novo Nordisk, Toronto, Canada) was injected intraperitoneally at a dose of 0.55 units/kg body wt into mice after a 5-h fasting period. Blood glucose was measured at indicated times following insulin administration.

**Islet Perifusion and Static GSIS Assays**
Batches of 50 mouse islets were stimulated with glucose in presence or absence of 10 nM GLP-1 (7-36)-amide (Bachem, Torrance, CA) as indicated. This assay was performed as reported previously (10) with secreted insulin in perifusate fractions determined by RIA (kit from Linco Research, St. Charles, MO). Results are presented as insulin secreted normalized to total insulin content of the perifused islets collected at the end of each assay.

GSIS assays from INS-1 cells were performed as described previously (9) (see Online Supplementary Methods). Insulin release and insulin content in INS-1 cell lysates were determined using a homogenous time-resolved fluorescence insulin assay (Cisbio) in accordance with the manufacturer’s instructions, on a PHERAstar plate reader (BMG Labtech, Ortenberg, Germany). Insulin levels were normalized to total insulin content.

**Confocal Immunofluorescence Microscopy**

Pancreatic islet β-cells were treated as indicated and fixed, then immunostained as described previously (10). Images were examined using a laser scanning confocal imaging system (LSM510, Carl Zeiss, Oberkochen, Germany). Measurement of fluorescent mean intensity was determined by NIS-Elements AR 3.0 (Nikon Inc, Japan).

**Electrophysiology**

Cell membrane capacitance (Cm) was estimated by Lindau-Neher technique, implementing a “Sine-DC” feature of the lock-in module (40 mV peak-to-peak at 500 Hz frequency) in whole-cell configuration as previously described (11). Recordings were conducted using an EPC10 patch clamp amplifier equipped with Pulse and X-Chart software programs (HEKA Electronik, Lambrecht, Germany). Exocytotic events were elicited by a train of ten 500-ms depolarization pulses (1-Hz stimulation frequency) from -70 mV to 0 mV. All recordings were performed at 30°C. β-cells were identified by their large size, measured electrophysiologically with an average
Cm >6 pF as previously reported (27). Intracellular solution contained (in mM): 125 cesium glutamate, 10 CsCl, 10 NaCl, 1 MgCl₂, 5 HEPES, 0.05 EGTA, 3 MgATP, pH 7.2. The extracellular solution consisted of (in mM): 118 NaCl, 5.6 KCl, 1.2 MgCl₂, 10 CaCl₂, 20 tetraethylammonium chloride, 5 HEPES, and 5 D-glucose, pH 7.4.

**Total Internal Reflection Fluorescence Microscopy (TIRFM) Imaging**

TIRFM imaging were acquired by a Nikon TE2000U TIRF microscope at 5-Hz with a 100-ms exposure time as described previously (10,28). A monolayer of primary mouse β-cells was infected with adenovirus encoding NPY-EGFP and further cultured in 24-h before performing TIRFM. Before image acquisition, cells were pre-incubated for 30-min in basal 2.8 mM glucose (in KRB buffer), and then stimulated with 16.7 mM glucose in presence or absence of 10 nM GLP-1 as indicated at a flow rate of 1 ml/min; or stimulated with 50 mM KCl (pH 7.4, in KRB buffer). Insulin granule mobilization and exocytosis were analyzed by Matlab (Math Works, Natick, MA), ImageJ (NIH), and Igor Pro (WaveMetrics, Lake Oswego, OR) softwares.

Fusion events indicated as flashes of fluorescence indicating NPY-EGFP cargo emptying were manually selected and assigned to one of three modes: pre-docked SGs that were visible before stimulation, no dock newcomers SGs that fused without remaining at the plasma membrane for <200-milliseconds (interval of one frame), and short dock newcomers SGs that appeared during stimulation and stably remained for >200-milliseconds before fusion occurs. An increase of EGFP fluorescence exceeding five times over the standard deviation of the fluorescence fluctuation was considered as fusion events. Fluorescence of individual SGs was measured as mean brightness of the defined circle (1.335 µm in diameter). The number of pre-docked SGs was counted and averaged at the first 2-min prior to stimulation.

**Islet Morphometry, Immunohistochemistry, and Immunofluorescence**
Seven-micron-thick sections was obtained as we described previously (29), then stained with insulin, glucagon (NovoCastra Laboratories, Leica Biosystems, Germany) or Ki67 antibodies (DAKO Diagnostics, Mississauga, Canada). Total β-cell area, α-cell area, islet size and islet number as well as total pancreatic area were determined on insulin-stained sections (10). Total β-cell area, α-cell area and total islet number were calculated per total pancreatic area. Immunofluorescent-stained sections were visualized using a Zeiss inverted fluorescence microscope.

**Statistical Analysis**

All data are presented as mean ± SEM. Statistical significance was assessed by repeated measure ANOVA or Student’s *t*-test in SigmaStat (Systat Software, Inc). Significant difference is indicated by asterisks (*p*<0.05, **p**<0.01).

**RESULTS**

**Syn-2 Deletion Increases Insulin Secretory Capacity and Improved Glucose Homeostasis But Has No Impact on β-cell Mass**

In pancreatic islets of WT mice (litter controls), Syn-2 colocalized mainly with β-cell insulin SGs (**Fig. 1A**), confirmed by subcellular fractionation (**Fig. 1B**), although small amounts of Syn-2 were in enriched PM fractions. In comparison, Syn-1A was most abundant in PM and Syn-3 in SGs. To begin to assess for a clinically-relevant role of Syn-2 in insulin secretion, we examined for presence of Syn-2 in normal and type-2 diabetes (T2D) human islets (**Fig. 1C**). We confirmed that Syn-1A levels in T2D human islets were reduced (by 55%) (30). T2D islet Syn-3 levels were normal, but surprisingly, islet Syn-2 levels were also normal. We then studied the role of Syn-2 in insulin secretion, with the initial thinking that Syn-2 plays a pro-fusion role in insulin SG
exocytosis, perhaps redundant to Syn-1A and/or Syn-3.

To assess for the endogenous role of Syn-2 in insulin secretion, we used a global Syn-2-KO mouse previously reported (14,15). Western blotting of islets from litter control WT and Syn-2-KO mice confirmed the absence of Syn-2, whereas levels of other exocytotic syntaxins, SNAP25, VAMPs and SM proteins were not altered by Syn-2 deletion (Fig. 1D). Syn-2, which shares >60% homology to Syn-1A (31), was expected to reduce in vivo insulin secretory capacity when depleted, similar to Syn-1A deletion (5). Surprisingly, IPGTT showed that Syn-2-KO mice paradoxically exhibited better glucose tolerance (Fig. 1E), with corresponding higher plasma insulin levels (Fig. 1F). To negate a possible influence by insulin-sensitive tissues on glucose homeostasis, insulin tolerance tests exhibited comparable insulin sensitivity (Fig. 1G; AUC in right panel); and weights of Syn-2-KO and WT mice used for in vivo studies were similar suggesting no difference in food intake (Fig. 1H).

We explored for the reasons underlying how global Syn-2 deletion could explain the higher GSIS in vivo in the Syn-2-KO mice. Since Syn-2 affected morphogenesis of several tissues, a plausible explanation is that Syn-2 affected β-cell mass or islet architecture, which we reported for VAMP8 to which Syn-2 binds (10). Islets of 10-week-old Syn-2-KO and WT mice did not show any difference in β-cell area per pancreatic area, number or size of islets, or Ki67-positive cells (Fig. S1A). Islet architecture was not altered with α-cells remaining in the islet mantle and α-cell mass per pancreatic area unchanged (Fig. S1A). These results exclude the possibility that Syn-2 depletion affects β-cell proliferation. A second explanation is that global Syn-2 deletion could affect intestinal release of incretin hormones glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) (32). In response to oral GTT (OGTT), fasting and peak plasma levels of total blood GLP-1 and GIP were not significantly different between WT and KO mice.
Here, we confirmed the better glucose tolerance and higher insulin levels in the Syn-2-KO mice (Fig. S1B). A third reason is alteration in the release of glucagon from islet α-cells. However, instead of hyperglucagonemia which would contribute to the observed hyperglycemia (33), we instead saw a mild reduction (only 17.5%) in fasting blood glucagon (Fig. S1C). This is likely not attributed to a primary effect of Syn-2 deletion on α-cell glucagon secretion, but more likely due to the slightly higher (but insignificant) fasting glucose levels observed during the overnight fast (Fig. 1F).

Taken together, the improved glucose homeostasis in the global Syn-2-KO mice must be due to an increase in insulin release from each β-cell, leading us to the hypothesis that Syn-2 must be playing a negative regulatory role in insulin secretion. A corollary hypothesis that is of clinical relevance is that the normal levels of Syn-2 in human T2D islets might contribute to the reduced effectiveness of the already low islet levels of Syn-1A (30) on predocked SG fusion (5). Conversely, reducing islet Syn-2 levels could potentially relieve Syn-2 inhibition on insulin SG fusion, which could increase either Syn-1A actions on predocked SG fusion (5) or Syn-3 actions on newcomer SG fusion (9). These effects of β-cell Syn-2 depletion could potentially rescue the deficient biphasic GSIS in T2D human islets. We therefore focused the rest of our study to examine how Syn-2 depletion in β-cell could promote insulin SG exocytosis.

**Syn-2 Deletion Enhanced Insulin Release by Increasing Priming and Mobilization of Insulin SG Pools**

Physiologic biphasic GSIS was assessed by employing islet perifusion assays whereby Syn-2-KO islets compared to WT islets showed a 110% increase in first-phase (first 15-min) and 114% increase in second-phase (next 25-min) 16.7 mM GSIS, and an even greater increase of 272% in 10 nM glucagon-like peptide (GLP)-1 potentiation of 16.7 mM GSIS (Fig. 2A, B). To
confirm that this potentiated secretory response was attributed to Syn-2 depletion per se, we performed a rescue study whereby Syn-2 KO islets were transduced with Ad-Syn-2-GFP (98% infection efficiency confirmed by GFP fluorescence, Fig. S2A) which reduced first-phase (by 48%) and second-phase GSIS (by 35%, Fig. 2B) to comparable levels as WT mouse islets.

Exocytotic capacity of single β-cells was assessed by patch clamp membrane capacitance measurements (Cm) using serial depolarization (Fig. 2C), wherein the first 2 pulses estimate the size of the RRP that was previously purported to account for much of first-phase GSIS from whole islets, and succeeding pulses (3rd–10th) measure RRP refilling purported to account for second-phase GSIS (34,35). Compared to WT β-cells, Cm increases in Syn-2 KO β-cells were increased at each depolarizing pulse (Fig. 2D, E), with the size of the RRP (ΔCm1st–2nd pulse) increased by 267% (23.5±7.1 fF/pF; WT: 6.4±1.5 fF/pF) and the rate of SG refilling (ΔCm3rd–10th pulse) increased by 172% (37.3±10.1 fF/pF, WT: 13.6±2.5 fF/pF). These results suggest that Syn-2 depletion increases depolarization-induced exocytosis by enhancing both priming and refilling of insulin SG pools.

Our previous work showed that Syn-1A and Syn-3 (36,37) directly bind and regulate calcium (Ca²⁺) channels, which raised the possibility that Syn-2 effects on insulin secretion might in part be due to possible actions on Ca²⁺ channels or intracellular Ca²⁺ release. Glucose (16.7 mM, Fig. S2B) and KCl (50 mM, Fig. S2C)-evoked intracellular Ca²⁺ release was not different between Syn-2-KO or WT mouse β-cells. Furthermore, Syn-2 deletion in mouse β–cells did not affect voltage-gated Ca²⁺ currents (Fig. S2D). These results taken together indicate that Syn-2 must be acting predominantly on the level of SG fusion.

**Depletion of Syn-2 Recruits More Newcomer SGs to PM to Undergo Exocytosis as well as Promotes Fusion of Predocked SGs**
Priming and refilling of SG pools have been attributed to sequential processes of docking of SGs onto PM, then priming, wherein predocked SGs sitting on PM for indefinite periods become ready for Ca\(^{2+}\)-triggered fusion. These processes revolve around assembly and disassembly of cognate SNARE and accessory proteins that bind Syn-1A (2). In fact, global deletion of Syn-1A reduced the number and fusion-competence of predocked insulin SGs (5). Now well-described is the population of newcomers SGs that undergo little to no residence time at the PM before fusion, which accounts for a much larger portion of secretion in both first- and second-phase GSIS (3,6,7,10). In fact, we had reported that newcomer SGs also contribute to the RRP as measured by patch clamp Cm (10). We employed time-lapse TIRF microscopy to track exocytosis dynamics of insulin SGs tagged by adenovirus transduction of neuropeptide Y (NPY)-EGFP. At unstimulated (2.8 mM glucose) state (Fig. 3A), punctate fluorescence indicating docked SGs were not different between WT (10.4±1.34/100 µm\(^2\)) and KO β-cells (11.7±0.70/100 µm\(^2\)). When stimulated with 16.7 mM glucose plus 10 nM GLP-1, assessment of cumulative fusion events over time (Fig. 3B) showed much more fusion events during the 20 min acquisition in Syn-2-KO β-cells.

We dissected the single SG fusion dynamics. At 2.8 mM glucose, we seldom found spontaneous fusion events. When 16.7 mM glucose was added after preincubation with 10 nM GLP-1 to potentiate insulin exocytosis, SG fusion events were observed as flashes of fluorescence that rapidly dissipate in cloud-like diffusion pattern. These exocytotic events were not uniform and could be categorized into three distinct modes (Fig. 3C) (10). Even in first-phase GSIS, newcomer SGs already accounted for >70% of exocytotic events in WT β-cells (top, Fig. 3D), which corresponded to the diverging cumulative increase in exocytosis in WT compared to Syn-2-KO β-cells (Fig. 3B). Enhancement of exocytosis in both phases of GSIS
evoked by 16.7 mM glucose plus GLP-1 was accounted for entirely from potentiation in recruitment and fusion of no-dock newcomer SGs (WT: 6.96±0.87 vs KO: 20.30±4.8/100 µm² in first-phase; WT: 5.16±1.20 vs KO: 10.80±2.61/100 µm² in second-phase) (Fig. 3E), as fusion of predock SGs were similar between WT and KO β-cells. There was no change in short-dock newcomer SGs. For a more critical assessment, we assessed exocytosis evoked by 16.7 mM glucose only, or by 50 mM K⁺ stimulation, which the latter causes fusions of predocked SGs predominantly. Glucose only stimulation caused similar trends as we saw with GLP-1-potentiated GSIS with a predominant increase in fusion of no-dock newcomer SGs (Figs. 3F, S3A). However, KCl stimulation showed a dramatic preferential enhancement of predocked SG fusion in Syn-2-KO β-cells (WT: 12.5±1.36 vs KO: 19.3±2.52/100 µm²) (Figs. 3G, S3B), consistent with the larger depolarization-evoked RRP (predominantly predocked SG) exocytosis in the Cm study (Fig. 2C-F). These results indicate that Syn-2 may be acting as a clamp that blocks recruitment and exocytosis of no-dock newcomer and predocked SGs (to lesser extent), which when relieved by Syn-2 depletion, then allows both no-dock newcomer and predocked SGs to proceed to fusion. These results are reminiscent of Syn-3 (9) and VAMP8 (10) over-expression which promoted recruitment and exocytosis of no-dock newcomer SGs, suggesting that Syn-2 might act as negative regulator (i.e. inhibitory SNARE) against Syn-3/VAMP8 and Syn-1A/VAMP2 pro-fusion complexes.

Absence of Syn-2 Enables Formation of More Pro-Fusion SNARE Complexes

Syn-1A forms a complex primarily with VAMP2, with the complete SM/SNARE complex including Munc18a and SNAP25, to mediate exocytosis of predocked SG (2). In contrast, the putative SM/SNARE complex that mediates newcomer SGs is formed by Munc18b (8), Syn-3 (9), VAMP8 (10) and SNAP25. Peculiarly in those studies, we also noted the formation of Syn-2
complexes with Munc18b and SNAP25, binding both VAMP2 and VAMP8. This led us to postulate that Syn-2’s inhibitory role in exocytosis might be as a negative regulator that binds the two VAMPs in competition with their respective pro-fusion Syn-1A (5) and Syn-3 (9). Since pancreatic islets provide insufficient protein required for co-immunoprecipitation (co-IP) assays, we employed two surrogates, endocrine adrenal glands of the same animals experimented above which have very similar profile of exocytotic proteins as pancreatic islets (Fig. 4A), and Syn-2 siRNA-treated insulinoma INSU1 cells. In WT mice, Syn-2 antibody co-IPed Munc18b, SNAP25 and SNAP23, and both VAMP2 and VAMP8 from adrenal gland lysates (Fig. 4B). While there was no change in adrenal gland protein levels of Syn-1A and Syn-3 SNARE/SM complexes, co-IP with Syn-1A and Syn-3 antibodies co-precipitated more of their respective SM/SNARE complexes from Syn-2 KO mice than WT mice adrenal glands (Fig. 4C, D). Specifically, Syn-2 deletion enabled Syn-1A to pull down more SNAP25 (76% increase) and VAMP2 (61% increase) (Fig. 4C); Syn-3 similarly pulled down more SNAP25 (63% increase) and VAMP8 (59% increase) (Fig. 4D).

INS-1, a β-cell surrogate we have used for such studies, upon stimulation with 16.7 mM glucose plus 10 nM GLP-1, activated formation of SM/SNARE complexes, whereby at basal state (0.8 mM glucose), SM proteins bind syntaxins in closed conformation that has reduced capacity to form SNARE complexes. We confirmed that Syn-2 antibody co-IPed the same proteins from stimulated INS-1 (8; 10), including VAMP2 and VAMP8 (Figs. 4E, S4D). We then depleted Syn-2 in INS-1 cells with siRNA by 72.2% at 48-hrs and 83.8% at 72-hrs (Figs. 4F, S4B) to assess whether this affects pro-fusion Syn-1A and Syn-3 SNARE complexes. We confirmed their effects on insulin secretion, which showed enhanced GSIS (16.7 mM glucose) in absence (by 51%) or presence of 10 nM GLP-1 (by 54%) (Figs. S4A,C). At basal state, Syn-1A
and Syn-3 in both Syn-2 siRNA- and scramble siRNA-treated INS-1 cells, bind similar amounts of cognate Munc18a and Munc18b, respectively, and similarly small amounts of SNAP25 (and SNAP23), but very little VAMPs were co-IPed (Fig. 4G, H). On stimulation, Syn-1A (Figs. 4G, S4E) and Syn-3 (Figs. 4H, S4F) bind similarly more SNAP25 and SNAP23. However, from Syn-2-depleted cells, Syn-1A pulled down more VAMP2 (by 93.4%), and Syn-3 pulled down more VAMP8 (by 81.7%) than scrambled siRNA-treated cells.

These adrenal gland (Fig. 4A-D) and INS-1 results (Fig. 4E-H) were very comparable, which taken together, indicate that endogenous Syn-2 reduces the capacity of Syn-1A and Syn-3 to form pro-fusion SM/SNARE complexes with VAMP2 and VAMP8, respectively, thus acting as an inhibitory SNARE (24) that blocks exocytosis mediated by these SM/SNARE complexes. To assess this more critically on whether Syn-2 competes against Syn-1A and Syn-3 for binding to v-SNAREs, Syn-2 was coexpressed with Syn-1A or Syn-3 in HEK cells and subjected to pull down with GST-VAMP2 or GST-VAMP8 (Fig. 5). Syn-2 potently reduced Syn-3 binding to cognate VAMP8 by 67.4%, providing the mechanism by which Syn-2 deletion in β-cell promoted newcomer SG fusion (Fig. 3) by increasing Syn-3 SM/SNARE complex formation (Fig. 4). Syn-2 also inhibited Syn-1A binding to cognate VAMP2 but more moderately by 35.2% (Fig. 5), suggesting that Syn-2 depletion could (albeit perhaps less effectively) promote fusion of predocked SGs. Thus, therapeutically inducing Syn-2 depletion in T2D β-cells would greatly potentiate newcomer SG fusion in both first- and second-phase GSIS since islet Syn-3 levels remained normal (Fig. 1C); and perhaps also enable the reduced levels of Syn-1A (Fig. 1C) to more effectively promote and at least partially restore predocked SG fusion in first-phase GSIS. Interestingly, Syn-2 also reduced Syn-3 promiscuous binding to VAMP2 by 55.9%, and Syn-1A promiscuous binding to VAMP8 by 43.5%.
DISCUSSION

Inhibitory SNAREs, demonstrated by in vitro lipid fusion assays to form non-fusogenic complexes, were postulated to provide a countercurrent fusion pattern within Golgi stacks (24); and subsequently shown in bacteria to block the fusion of infectious phagosomes with endocytic compartments containing degradative enzymes (38). We here demonstrated this concept of inhibitory SNAREs to be applicable to mammalian cells in modulating insulin exocytosis that profoundly influences whole animal glucose homeostasis. This apparent deliberate mechanism to reduce efficiency of exocytosis adds to the plasticity of some secretory cells in which a slower secretory process (than neurons) of hormone release is required to respond to nutrient absorption. Along this thinking, it is possible that SynU2 might not be a pure inhibitory SNARE per se but rather an inefficient fusion protein that serves the extremely slow exocytosis in some non-neuronal cells such as pancreatic acinar cells (18) to enable metered release of digestive enzymes over several hours required to complete food digestion after a meal. Syn-2’s role as a pro- or inhibitory SNARE may thus be cell-context-specific; and in islet β-cells, Syn-2 might be a decelerator t-SNARE that brakes the efficient fusogenic Syn-1A and Syn-3. A release of the Syn-2 ‘brake’ in secretory-deficient T2D β-cells exhibiting reduced levels of pro-fusion SNARE proteins (1,3,30) could be a therapeutic strategy to improve GSIS in diabetes.

Syn-2 depletion seems to have strong effects on increasing newcomer SG fusion mediated by Syn-3 (9) after glucose (and GLP-1-potentiated) stimulation, which is explained at least in part by the stronger affinity of Syn-2 than Syn-3 for newcomer SG v-SNARE VAMP8 (10). Syn-2 depletion resulted in lesser increase in fusion of predocked SGs mediated by Syn-1A, unless selectively-evoked by high KCl concentration. The latter may be because the pool of primed
predocked SGs are limited in normal β-cells (even less in diabetic β-cells), which may already be saturated with pre-assembled Syn-1A SM/SNARE complexes with Syn-1A having a higher affinity for predocked SG v-SNARE VAMP2 than Syn-2. KCl stimulation, more likely to recruit additional Syn-1A SM/SNARE complexes than glucose stimulation, may explain its receptiveness to Syn-2 depletion. The much bigger pool of newcomer SGs requires continuous new assembly of Syn-3 SM/SNARE complexes to sustain second-phase GSIS (and GLP-1 potentiation), which is more receptive to Syn-2 depletion. β-cell Syn-2 depletion thus presents a favorable target for therapeutic intervention in combination with incretin or sulfonylurea treatments, particularly in the background of reduced SNARE protein levels in diabetic β-cells (30).

More work will be needed to elucidate the putative domains within Syn-2 that confer its feature of slower fusion or fusion block compared to Syn-1A and Syn-3, which share >60% amino acid identity with Syn-2 (31). Such comparative structural-function insights between these syntaxins could reveal the distinct features of Syn-1A mediating the prerequisite SG docking which Syn-3 seems to circumvent in mediating fusion of newcomer SGs with minimal residence time at the PM. Perhaps the SNARE complexes formed by the three syntaxins ‘zipper up’ very differently when mediating membrane fusion (39,40), and maybe with Syn-2, the zipper somehow gets ‘stuck’.
AUTHORS’ CONTRIBUTION

H.Y.G. and D.Z. formulated the original hypothesis; then on the manuscript revision, L.X. also helped in drafting the manuscript. Co-authors who performed the experiments include: D.C.R made the Syn-2 KO mouse; electrophysiology by L.X., protein-binding and western blotting studies by S.D., Y.K. and H.X., TIRFM imaging by D.Z, confocal imaging by D.Z. and T.L., animal experiments by D.Z. and J.B.H., and islet perfusion assays by D.Z. and T.Q. L.O., D.C.R. and H.Y.G. contributed to the Discussion. All authors discussed the results. H.Y.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. We thank E. Kwan and X. Lin for technical assistance.

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DUALITY OF INTEREST

No potential conflicts of interest relevant to this article were reported.
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FIGURE LEGENDS

Figure 1-Syn-2 deletion improves GSIS and glucose homeostasis. **A:** Representative immunofluorescence images of Syn-2 (green) with insulin (red) in mouse pancreatic β-cells, with their colocalization shown in merge images (yellow). Shown are representative images of three independent experiments. Scale bars, 5 µm. **B:** Subcellular distribution (plasma membrane (PM, 20 µg protein), cytosol (20 µg) and secretory granules (SG, 20 µg)) of Syn-1A, -2, -3 and their cognate SM proteins in INS-1 cells. Na+/K+-ATPase, GAPDH and insulin were used as positive markers for PM, cytosol and SG, respectively to verify the purity of each fraction. For analysis (N=3, bottom), the level in each fraction was expressed as a percentage of the signal of the strongest (maximum) band. **C:** Western blot (top) and analysis (N=3, bottom) of syntaxin isoforms levels in normal and T2D human pancreatic islets (25 µg protein). Rat brain (5 µg) used as positive controls. **D:** Western blot of SNARE and SNARE-associated protein levels in pancreatic islets of WT and Syn-2 KO mice (25 µg protein). Rat brain (5 µg) used as positive and negative controls. For analysis (N=3, bottom), the level of each protein was normalized to the tubulin band. **E-F:** IPGTTs were performed with blood glucose levels in **E** and insulin secretion in **F** assessed during IPGTT. Insulin secretion shown as AUC (area under the curve) encompassing 120 min of IPGTT (right). N=19 per group. **G:** Blood glucose levels during IPITT and corresponding AUC encompassing 120 min of IPGTT (right). N=8 per group. **H:** Weights of WT and Syn-2 KO mice used to perform IPGTT or insulin tolerance tests. Results are shown as mean ± SEM. *p<0.05; **p<0.01.

Figure 2-Syn-2 deletion increases biphasic insulin secretion. **A:** Islet perifusion assays in WT and Syn-2-KO mice pancreatic islets (left) and corresponding AUCs of insulin release (right)
stimulated by 16.7 mM glucose and then with application of GLP-1 (10 nM). Data are from 6 independent experiments. **B**: Islet perifusion assays of Syn-2-KO mouse islets whose Syn-2 expression is restored by Ad-Syn-2-eGFP infection (see **Fig. S2A**); corresponding AUCs shown on the right. **C**: Representative recordings of ΔCm during of a train of 500 ms depolarizations from -70 mV to 0 mV in WT (black) and Syn 2 KO (grey) β-cells. **D-E**: Changes in cell Cm for each pulse in **D** and cumulative changes in Cm normalized to basal cell Cm (fF/pF) in **E** during a train of ten 500-ms depolarizations in WT (n=20 cells) and Syn-2 KO (n=18 cells) β-cells. **F**: Summary of Cm evoked by the first two pulses (pulses 1-2 represent the RRP) and next eight pulses (pulses 3–10 represent SG mobilization). n=18–20 cells. Summary graphs shown as mean ± SEM. *p<0.05.

**Figure 3-Syn-2 deletion increases newcomer SG exocytosis during biphasic GSIS. A**: TIRF microscopy images of docked insulin SGs in WT or Syn-2 KO β-cells (*left*). Scale bars: 2 µm. Averaged SG densities before stimulation (*right*). **B**: Cumulative insulin SG fusion events per cell per 100 µm$^2$ stimulated as indicated. **C**: Kymographs and corresponding fluorescence intensity curves showing three modes of insulin SG fusion events: pre-dock (black bar), newcomer-no dock (open bar), and newcomer-short dock (gray bar). **D**: Histogram of fusion events in first (first 5 min after 16.7 mM glucose plus 10 nM GLP-1 stimulation) and second phases (5–18 min) in WT versus KO β-cells. Data obtained from four independent experiments (3-5 cells from each experiment). **E-F**: Summary of the three modes of fusion events in first (*left*) and second phases (*right*) stimulated by 16.7 mM glucose plus 10 nM GLP-1 in **E** or by 16.7 mM glucose in **F**. Corresponding histogram of fusion events for **Fig. 3F** seen in **Fig. S3A**. **G**: Summary of predocked and newcomer fusion events stimulated by 50 mM KCl.
Corresponding histogram of fusion events seen in Fig. S3B. Summary graphs shown as mean ± SEM. *p<0.05; **p<0.01

**Figure 4**-Syn-2 deletion enables formation of more pro-fusion SM/SNARE complexes. **A:** SM/SNARE protein profile of adrenal glands from WT and Syn-2 KO mice. **B:** Syn-2 co-IP of cognate SM/SNARE proteins in WT adrenal gland lysates. Representative of 2 experiments. **C** and **D:** Co-IP of Syn-1A in **C** or Syn-3 in **D** pulled down SM/trans-SNARE complexes from WT vs Syn-2 KO mice adrenal glands. Representative of 3 independent experiments with samples performed in duplicate; respective densitometry analyses shown in bottom panels. Values from WT adrenals were used as Control (100%), wherein all other values were normalized to control. **E:** Syn-2 Co-IP of cognate SNARE/SM proteins in control INS-1 cells, treated as indicated; analysis in **Fig. S4D. F:** Syn-2 siRNA knockdown in INS-1 cells, compared to scrambled siRNA. Representative of 3 experiments; analysis in **Fig. S4A,B** (proteins levels) and **Fig. S4C** (insulin secretion). **G** and **H:** Co-IP of Syn-1A in **G** or Syn-3 in **H** pulled down cognate SNARE/SM proteins in Syn-2 si-RNA vs control scrambled nonsense siRNA-transduced INS-1 cells, treated as indicated. Representative of 3 independent experiments with samples performed in duplicate, analysis in **Fig. S4E, F.**

**Figure 5**-Syn-2 blocks Syn-3 and Syn-1A binding to VAMP8 and VAMP2. **A:** HEK cells were transfected with Syn-1A, Syn-2 or Syn-3 alone, or Syn-2 cotransfected with Syn-1A or Syn-3. 48 hours after transfection, cells were collected and GST (as negative control), GST-VAMP2 or GST-VAMP8 was used to pull down Syn-1A, Syn-2 and Syn-3 from the cell lysate extracts. Shown is a representative of three separate experiments, whereby the percentage
recovery of total protein for Syn-1A alone is 3.02±0.56%, and Syn-3 alone is 2.88±1.01%.

B. Analysis of the three experiments. For direct comparison of the 3 experiments, the results were normalized to the percentage of Syn-A or Syn-3 use alone (Control) performed in each experiment.
Diabetes
A

| Syn-2 | + | - | - | - |
| Syn-1 | - | - | - | - |
| Syn-3 | - | - | + | - |

GST pull down

3% input

GST | GST-VAMP2 | GST-VAMP8
--- | --- | ---
35 | 35 | 35
35 | 35 | 35
35 | 35 | 35

B

% of control

| GST-VAMP2 | GST-VAMP8 | GST-VAMP2 | GST-VAMP8 |
|---|---|---|---|
| Syn-1A | Syn-1A | Syn-1A | Syn-1A |
| Syn-2 | Syn-2 | Syn-2 | Syn-2 |
| Syn-2 | Syn-2 | Syn-2 | Syn-2 |

169x197mm (300 x 300 DPI)
Figure S1-Syn-2 deletion does not affect islet morphology, β-cell mass, or GLP-1/GIP secretion.  

**A:** Left: Insulin-immunostained pancreatic sections. Scale bars, 600 µm; Right: Ratios of β-cell area per pancreatic area on mouse pancreatic sections. Bottom from left to right: Ki67-positive islet β-cells as percentage of total islet β-cells on mouse pancreatic sections; Ratios
of α-cell area per pancreatic area; islets numbers per pancreatic area; islet sizes on pancreatic sections. N=8-10 mice for each. **B**: Circulating blood levels of total GLP-1 (*left*) and GIP (*Right*) immunoreactivity (*top*) were measured before and after oral glucose administration, along with determination of blood glucose and insulin levels (*bottom*). N = 6 for each group. **C**: Fasting plasma glucagon levels of WT and Syn-2 mice (N = 3). *p < 0.05; ***p < 0.001. Summary graphs shown as means ± SEMs.
Figure S2-related to Figure 2. A: Confocal images of Syn-2-KO mouse islet β-cells whose Syn-2 expression was restored by Ad-Syn-2-eGFP infection. Top: Syn-2-KO islet β-cells infected with Ad-Syn-2-eGFP; Bottom: Syn-2 KO islet β-cells infected with Ad-eGFP as control. Scale bar: 50 µm. B-C: Calcium imaging with fluo-4 showed there was no difference in intracellular Ca\(^{2+}\) concentration increase between WT and Syn-2-KO mouse β-cells stimulated with high glucose (B, 16.7 mM) or with high K\(^+\) (C, 40 mM) stimulation. D: Deletion of endogenous Syn-2 in mouse β-cells did not affect voltage-gated calcium channel currents. Representative traces showing Cav currents recorded in whole-cell mode from -70 to 70 mV with 10-mV increment in WT and Syn-2 KO mice (Left). Current-voltage relationship of Cav\(_{\text{v}}\)
from WT (n=15) and Syn-2 KO (n=11) β-cells (right). Current were normalized to cell capacitance to yield current density. Values are means ± SEMs.

Figure S3—Syn-2 deletion increases newcomer SG exocytosis stimulated by 16.7 mM glucose and pre-dock SG exocytosis stimulated by 50 mM KCl. **A**: Histogram of fusion events in first (first 5 min after 16.7 mM glucose stimulation) and second phases (5–18 min) in WT (top) versus Syn-2-KO β cells (bottom). Three patterns of fusion events (pre-dock, newcomer-no dock, newcomer-short dock) are indicated by blue, red and green bars, respectively. Data obtained from five independent experiments (2-4 cells from each experiment). **B**: Histogram of fusion events during 200 seconds of acquisition in WT (top) versus Syn-2-KO β cells (bottom). Pre-dock and newcomer SG fusion events are indicated by blue and red bars, respectively. Data obtained from three independent experiments (3-5 cells from each experiment).
Figure S4-Data analysis for Figure 4. A: Western blot analysis of Syn-2 knockdown expression in INS-1 cells. INS-1 cells were transfected with Syn-2 siRNA and the scrambled siRNA were used as controls. B: Analysis of Western blots in Fig. 4F of siRNA knockdown of Syn-2 expression in INS-1 cells at 48 and 72 hrs. C: Glucose (as indicated) with or without the presence of 10 nm GLP-1 stimulated insulin secretion were performed on Syn-2 siRNA knockdown and scrambled control INS-1 cells. Data from 4 independent experiments. D-F: Quantitative analysis of indicated co-IPed proteins by Syn-2 (D for Fig. 4E, untreated INS-1), Syn-1A (E for Fig. 4G, Syn-2 siRNA-treated INS-1) and Syn-3 (F for Fig. 4H, Syn-2 siRNA-treated INS-1) antibodies. Summary graphs shown are means ± SEMs, N=3. *p< 0.05 per Student’s t test; NS, no significant difference.