New Roles of Syntaxin-1A in Insulin Granule Exocytosis and Replenishment

In type-2 diabetes (T2D), severely reduced islet syntaxin-1A (Syn-1A) levels contribute to insulin secretory deficiency. We generated β-cell-specific Syn-1A-KO (Syn-1A-βKO) mice to mimic β-cell Syn-1A deficiency in T2D. Glucose tolerance tests showed that Syn-1A-βKO mice exhibited blood glucose elevation corresponding to reduced blood insulin levels. Perifusion of Syn-1A-βKO islets showed impaired first- and second-phase glucose-stimulated insulin secretion (GSIS) resulting from reduction in readily releasable pool and granule pool refilling. To unequivocally determine the β-cell exocytic defects caused by Syn-1A deletion, EM and total internal reflection fluorescence microscopy showed that Syn-1A-KO β-cells had a severe reduction in the number of secretory granules (SGs) docked onto the plasma membrane (PM) at rest and reduced SG recruitment to the PM after glucose stimulation, the latter indicating defects in replenishment of releasable pools required to sustain second-phase GSIS. Whereas reduced predocked SG fusion accounted for reduced first-phase GSIS, selective reduction of exocytosis of short-dock (but not no-dock) newcomer SGs accounted for the reduced second-phase GSIS. These Syn-1A actions on newcomer SGs were partly mediated by Syn-1A interactions with newcomer SG VAMP8.

Pancreatic β-cells release insulin in a biphasic pattern (1, 2). Exocytosis of several pools of insulin secretory granules (SGs) mediated by distinct membrane fusion machineries underlie each of the two phases of glucose-stimulated insulin secretion (GSIS) (1, 2). The fundamental components of membrane fusion machinery are three SNARE proteins (syntaxin, SNAP-25, synaptosome-associated protein of 25 kDa), and VAMP (vesicle-associated membrane protein) and nSec/Munc18 (SM) protein, which act to remodel and activate the SNARE complex assembly (3). Each vesicle SNARE (v-SNARE) (VAMP) and target membrane SNARE (t-SNARE) (syntaxins, SNAP25) and SM protein constitute a family of isoforms (4) to enable combinatorial matching of cognate partners that underlie the molecular basis of distinct exocytotic events (4). Interestingly, β-cells employ almost all of the major SM-SNARE complexes to mediate exocytosis of distinct insulin SG pools. The current thinking is that Munc18a-Syn-1A-VAMP2 complex mediates the pool of insulin SGs that dock onto plasma membrane (PM) for an indefinite period, called “predocked” SGs, until a strong Ca2+ stimulus evokes fusion (5, 6). This pool of predocked SGs accounts for the readily releasable pool mediating first-phase GSIS (1, 2). A much larger number of insulin SGs called “newcomer” SGs could be mobilized from the cell interior to undergo fusion but were noted to take only minimal to no residence time at the PM (7, 8). Newcomer SGs account for almost all of second-phase GSIS and a substantial amount of first-phase GSIS (7, 8). We recently identified the SM-SNARE complex mediating newcomer fusion of Munc18b-Syn-3-VAMP8 (9–11). A smaller population of insulin SGs undergo homotypic SG-SG (compound) fusion (2), which can be potentiated by cAMP-acting glucagon-like peptide 1 (GLP-1) (12) and Ca2+-acting carbachol (13). SG-SG fusion, mostly in the form of orderly sequential SG-SG fusion in β-cells, is mediated by Munc18b-Syn-3-SNAP25 complex (9, 10, 14). The remaining SM-SNARE complex, Munc18c-Syn-4, was initially postulated to mediate biphasic GSIS by acting on predocked SGs in a manner redundant to Munc18a-Syn-1A (15, 16). Our recent work showed that Munc18c-Syn-4 also acts on newcomer SGs (17, 18). Furthermore, unlike β-cells, which employ redundant SM-SNARE complexes for exocytosis, Munc18c-Syn-4 in complex with VAMP2 and SNAP23 is the only SM-SNARE complex mediating glucose uptake in insulin-sensitive tissues, adipocyte, and muscle (15).

The strongest evidence for the role of Syn-1A in insulin exocytosis has been provided by the study employing a global Syn-1A knock-out (KO) mouse (6), originally generated to examine neuronal plasticity, but it showed remarkably few neuronal phenotypic abnormalities (19). Instead, Syn-1A-KO mice showed profound defects in insulin exocytosis (6), specifically much fewer predocked SGs that were fusion-incompetent, and apparently without perturbation in newcomer SGs. This is of
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clinical relevance because levels of Syn-1A and cognate proteins are severely reduced in islets of T2D patients, postulated to contribute to insulin secretory deficiency (20). However, the global Syn-1A deletion can potentially affect neuronal and endocrine secretions that might influence β-cell function. In fact, recent reports by the original group that created the mouse demonstrated a perturbation in the hypothalamic-pituitary-adrenal axis affecting corticosterone (21) and catecholamine release (22), two hormones that profoundly affect glucose homeostasis by their actions on insulin-sensitive tissues and secretion of islet hormones. Syn-1A is also present in α-cells to mediate glucagon secretion (23), which in turn might have paracrine influences on β-cells.

It therefore behooves us to unequivocally reassess the role of Syn-1A in β-cell insulin exocytosis per se, employing a β-cell-specific KO mouse (Syn-1A-βKO). Our results confirmed that Syn-1A deletion in β-cells caused a reduction in number and fusion of predocked SGs, resulting in reduced first-phase GSIS. Unexpectedly, Syn-1A deletion reduced SG replenishment to releasable pools after stimulation and selective reduction in fusion of short-dock newcomer SGs; both underlie the reduced second-phase GSIS. This reduced biphasic GSIS in vivo resulted in hyperglycemia.

Results

Generation of β-Cell-specific Syn-1A Knock-out (Syn-1A-βKO) Mice—Correct targeting was confirmed in 14 independent R1 ES cell clones by Southern blotting analysis (Fig. 1A), and two of these lines produced male chimeras for germ line transmission after aggregation. Mice that were heterozygous for floxed Syn-1A allele and heterozygous for RIP-Cre recombinase (Syn-1AfloX/−Cre+ ) were then crossed with heterozygous floxed Syn-1A mice (Syn-1AfloX/+Cre−) to generate litters that contained pups where the RIP-Cre had mediated excision of exons 2 and 3 of Syn-1A specifically in β-cells (Syn-1AfloX/floXCre+). The two control mice included mice that were homozygous for floxed Syn-1A allele but did not express Cre (Syn-1AfloX/floXCre−) and RIP-Cre mice (Syn-1A+/*Cre+) (Fig. 1A).

There is concern that some rat insulin 2 promoter (RIP)-Cre strains, such as Tg(Ins2-Cre)Mgi, exhibit leaky expression of Cre in the mid-brain and ventral brain regions, including the hypothalamus (24, 25). This is important because Syn-1A deletion in hypothalamic neurons can potentially perturb glucose homeostasis (26). We therefore chose another RIP-Cre strain, Tg(Ins2-Cre)Herr (27), which was reported to have a more restricted and punctate pattern of Cre expression in the brain, particularly in the hypothalamus, compared with the robust Cre leakage in other RIP-Cre strains, including the RIP-CreMgi and RIP-Cre/ERT. First, we confirmed this Tg(Ins2-Cre)Herr strain by sequencing of the specific 660-bp rat Ins2 promoter. Next, we confirmed that there was no or minimal brain leakage by performing total RNA analysis by quantitative PCR of the hypothalamus of our Syn-1A-βKO and RIP-Cre control mice using a sensitive quantitative RT-PCR protocol with high dynamic range. Hypothalamic expression levels of Cre were extremely low (supplemental Fig. S1A), with threshold (Ct) values (34.65 ± 1.13) below the cut-off C, value of 30 for positive gene expression in both groups (supplemental Fig. S1B). Consistent with the extremely low Cre expression level, hypothalamic Syn-1A levels were equivalent in both mouse groups, indicating that no deletion of Syn-1A occurred in Syn-1A-βKO mouse hypothalamus. There was also no difference in expression in the Syn-1A cognate partners, including Munc18a, VAMP2, and SNAP25, or non-cognate SNARE proteins Syn-3 and VAMP8 (supplemental Fig. S1A).

Islets were isolated from Syn-1A-βKO and control mice (12–14 weeks old) and subjected to Western blotting, which confirmed the reduction in Syn-1A levels without any effect on the levels of cognate Munc18a, VAMP2, and SNAP25 or non-cognate SNARE proteins (Fig. 1B). The residual Syn-1A in Syn-1A-βKO islets was due to Syn-1A present in non-β-cells shown on confocal microscopy. Here, in control mouse islets, Syn-1A localized to insulin-positive β-cells as well as glucagon-positive α-cells and somatostatin-positive δ-cells (Fig. 1C). In Syn-1A-βKO islets, Syn-1A was no longer present in insulin-positive β cells but still positive in glucagon-positive α-cells and somatostatin-positive δ-cells (Fig. 1D).

To ensure that any defects on β-cell insulin secretion are attributable to Syn-1A effect per se, we assessed for possible changes in β-cell mass (supplemental Fig. S2A) by several parameters (β-cells per pancreatic area, islet number and size), which showed no differences between the three mouse groups. We also assessed serum glucagon levels (supplemental Fig. S2B), which were not different between the Syn-1A-βKO mice and the two groups of control mice, consistent with the intact Syn-1A in the Syn-1A-βKO mouse α-cells (Fig. 1D).

Syn-1A-βKO Mice Are Glucose-intolerant from the Reduced Insulin Secretion—There is debate on whether RIP-Cre strains exhibit some glucose intolerance (28, 29). We therefore employed both Syn-1AfloX/floX and RIP-Cre mice as controls. An intraperitoneal glucose tolerance test (IPGTT) showed that RIP-Cre mice exhibited mild (Fig. 2Ai) but not significant reduction in glucose tolerance compared with Syn-1AfloX/floX mice (AUC analysis in Fig. 2A, ii). In contrast, Syn-1AβKO mice exhibited higher glycemic levels than either Syn-1A flox or RIP-Cre control mice (Fig. 2A, i and iii (AUC determined above basal levels)). This was caused by much reduced plasma insulin levels in Syn-1A βKO mice (Fig. 2A, ii and iii (AUC determined above basal levels)), encompassing the first-phase peak and second-phase plateau responses, when compared with control Syn-1AfloX/floX and RIP-Cre mice (the two controls were not significantly different from each other (Fig. 2A, ii and iii)). Basal insulin levels were also lower in the Syn-1A-βKO mice compared with the control mice. The weights of Syn-1A-βKO mice (32.5 ± 3.1 g) were not different from those of control mice (RIP-Cre control, 31.5 ± 3.3 g; Syn-1A flox control, 31.2 ± 2.6 g) (Fig. 2B). We also examined the fed and fasting glucose and insulin levels (Fig. 2C). After an 18-h overnight fast, Syn-1A βKO mice had higher glucose (8.4 ± 1.1 mmol/liter; RIP-Cre control, 6.1 ± 1.12 mmol/liter; Syn-1A flox control, 5.4 ± 0.8, mmol/liter) and lower insulin levels (0.44 ± 0.08 ng/ml; RIP-Cre control, 0.68 ± 0.09 ng/ml; Syn-1A flox control, 0.72 ± 0.07 ng/ml). In the fed state, Syn-1A βKO mice exhibited a higher rise in glucose (11.2 ± 0.9 mmol/liter; RIP-Cre control, 9.02 ± 0.7 mmol/liter; Syn-1A flox control, 8.5 ± 1.1 mmol/liter).
liter) with a corresponding lower increase in insulin levels (0.69 ± 0.11 ng/ml; RIP-Cre control, 1.24 ± 0.14 ng/ml; Syn-1A flox control, 1.21 ± 0.18 ng/ml). The net increase of fed over fasting insulin levels was much higher in the control than Syn-1A KO mice (Fig. 2C, ii).

**Syn-1A-KO Mouse Islets Exhibit Reduced Biphasic GSIS—**

To examine the direct contribution of pancreatic islet insulin secretion to physiologic glucose stimulation, we performed islet perfusion assays (Fig. 3A). Syn-1A deletion in β-cells resulted in reduction of both first-phase (AUC determined above basal levels; Fig. 3B; Syn-1A-KO, 1.98 ± 0.2 (ng/μg total insulin content) × min; Syn-1A flox control, 4.78 ± 0.59 (ng/μg total insulin content) × min; RIP-Cre control, 3.92 ± 0.26 (ng/μg total insulin content) × min) and second-phase (AUC determined above basal levels; Syn-1A-KO, 1.74 ± 0.32 (ng/μg total insulin content) × min; Syn-1A flox control, 4.41 ± 0.42...
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A i

Blood Glucose (mmol/L) vs Time (min)

- Syn-1A KO (N=11)
- RIP-Cre control (N=5)
- Syn-1A flox control (N=7)

A ii

Blood Insulin (ng/mL) vs Time (min)

- Syn-1A KO (N=11)
- RIP-Cre control (N=5)
- Syn-1A flox control (N=7)

B

Blood Glucose AUC vs Blood Insulin AUC

- Syn-1A KO (N=11)
- RIP-Cre control (N=5)
- Syn-1A flox control (N=7)

C i

Blood Glucose (mmol/L)

- Fasting
- Fed

- Syn-1A KO (N=11)
- RIP-Cre control (N=11)
- Syn-1A flox control (N=11)

C ii

Blood Insulin (ng/mL)

- Fasting
- Fed

- Syn-1A KO (N=11)
- RIP-Cre control (N=11)
- Syn-1A flox control (N=11)

FIGURE 2. Syn-1A-KO mice exhibit glucose intolerance because of reduced blood insulin levels. A, Syn-1A-KO mice are glucose-intolerant because of reduced insulin release into the circulation. IPGTTs were performed on Syn-1A-KO (n = 11) versus control mice (RIP-Cre (n = 5) and Syn-1A flox (n = 7)), from which we obtained blood glucose (i) and plasma insulin levels (ii), iii, corresponding AUCs determined above basal levels for glucose (i) and insulin (ii) because basal levels are different between experiments. B, weights of Syn-1A-KO mice (n = 20) versus control mice (RIP-Cre (n = 19) and Syn-1A flox (n = 17)) were not different. C, Syn-1A-KO mice (n = 14) showed higher blood glucose levels (i) and lower insulin levels (ii) than control mice (RIP-Cre (n = 11) and Syn-1A flox (n = 11)) after an 18-h fast and during the fed state. Results are shown as means ± S.E. NS, not significant. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Deletion of Syn-1A in Mouse β-Cells Reduces the Number of SGs Docked on the PM and SG Replenishment after Stimulation—Reduction of first-phase GSIS can be predicted to be due to reduction of SG docking and/or fusion of predocked SGs with the PM. To examine SG docking at the PM, we performed EM morphometric analysis on Syn-1A-KO β-cells compared with control (Syn-1A flox and RIP-Cre) β-cells at basal and after a 15-min 16.7-mmol/liter glucose stimulation (Fig. 4A), the latter to assess SG recruitment to PM to replenish releasable pools after depletion caused by first-phase release. We first ruled out any effect of Syn-1A deletion on insulin SG biogenesis in that the diameters of SGs between the three groups were similar (Fig. 4B), and there was no difference in SG densities in basal conditions or after stimulation (Fig. 4C). Predictably, Syn-1A-KO β-cells showed a large reduction of ~80% in the number of docked SGs in basal conditions (Fig. 4D; Syn-1A-KO, 0.2 ± 0.06; Syn-1A flox control, 0.91 ± 0.21; RIP-Cre control,
1.05 ± 0.24), which would explain the reduced first-phase secretion, consistent with the results of the global Syn-1A-KO mouse (6). After 15-min glucose stimulation of control β-cells to secrete and deplete the releasable pools, there was sufficient recruitment of SGs to the PM to replace the exocytosed SGs and sustain second-phase GSIS. However, glucose stimulation of Syn-1A-KO β-cells caused an even greater reduction (than basal conditions) of 95% in morphologically docked SGs (Syn-1A-KO, 0.06 ± 0.02; Syn-1A flox control, 1.23 ± 0.29; RIP-Cre control, 1.35 ± 0.33) (Fig. 4, A and D). Of note, glucose stimulation also caused a larger clearing of SGs further into the cell interior in Syn-1A-KO β-cells (see image 3 in Fig. 4A) compared with basal conditions. This was assessed by detailed analysis in Fig. 4E showing that Syn-1A-KO β-cells had a mild reduction in the number of SGs within 0–0.2 μm from the PM under basal conditions, which became very severe after glucose stimulation. In fact, this severe reduction in SGs also occurred within the deeper 0.2–0.4-μm concentric shell. Further into the cytoplasm from 0.4 to 1 μm, there was a larger accumulation of SGs in Syn-1A-KO β-cells compared with control β-cells. These results indicate that Syn-1A depletion reduced the mobilization of SGs from the cell interior to the PM required to replenish the releasable pools (see Fig. 5) that sustain second-phase GSIS (Fig. 3). This action of Syn-1A on recruitment of SGs to the PM is novel.

**Syn-1A-KO Mouse β-Cells Exhibit Reduced Priming and Mobilization of Insulin SG Pools**—We therefore then assessed for the effects of Syn-1A deletion on releasable insulin SG pools by employing patch-clamp membrane capacitance (Cm) measurements of single β-cells. Insulin SG exocytosis was elicited...
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A

Syn-1A flox control | RIP-Cre control | Syn-1A βKO

2.8 mM Glucose

PM

16.7 mM Glucose

PM

1

2

3

B

C

D

E

% of SGs density per μm²

Distance from the SGs center to the PM (μm)

0 50 100 150 200

0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6

0 2.8 mM G 16.7 mM G

NS

NS

NS

2.8 mM G 16.7 mM G

No. of SGs per mm²

0 2.8 mM G 16.7 mM G

No. of docked SGs per μm²

0 2.8 mM G 16.7 mM G

Syn-1A flox control

RIP-Cre control

Syn-1A βKO

Flop control 2.8 mM G

RIP-Cre control 2.8 mM G

Syn-1A βKO 2.8 mM G

Flop control 16.7 mM G

RIP-Cre control 16.7 mM G

Syn-1A βKO 16.7 mM G

NS

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NS

2.8 mM G 16.7 mM G

2.8 mM G 16.7 mM G

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2.8 mM G 16.7 mM G

2.8 mM G 16.7 mM G
FIGURE 4. Syn-1A deletion in mouse β-cells impairs insulin SG docking onto the plasma membrane at rest and recruitment to plasma membrane during stimulation. A and B, representative EM images of islets at basal conditions (2.8 mmol/liter glucose, top panels) and after stimulation (16.7 mM glucose for 15 min, middle panels) from control (Syn-1A flox, RIP-Cre) and Syn-1A-βKO mice from three independent experiments. The numbered bottom panels are enlarged views of the indicated areas in the middle panels. Scale bar, 500 nm. Black arrows, PM; white arrowheads, docked insulin SGs, which are almost absent in Syn-1A-βKO β cells at basal and stimulated conditions. B, diameter of SGs. C, number of SGs/μm² of cytoplasmic area. D, number of morphologically docked insulin SGs/μm of PM at their shortest distance of <50 nm from PM were qualified as morphologically docked SGs (white arrowheads in A numbered panels). B–D, total number of SGs counted from three independent experiments: Syn-1A-βKO, 2.8 mM glucose, 13 micrographs, 3376 SGs; 16.7 mM glucose, 17 micrographs, 4224 SGs; Syn-1A flox control, 2.8 mM glucose, 13 micrographs, 3376 SGs; 16.7 mM glucose, 19 micrographs, 4732 SGs. Values represent mean ± S.E. (error bars). 

FIGURE 5. Syn-1A deletion in mouse β-cells reduces the priming and mobilization of insulin SG pools. A–D, exocytosis in single islet β-cells (identified by Cm ≥ 10 picofarads) of Syn-1A-βKO versus Syn-1A flox control mice. A and B, representative recordings of exocytosis during a train of 500-ms depolarizations from −70 to 0 mV in Syn-1A flox control (A) and Syn-1A-βKO (B) β-cells. C, cumulative changes in cell capacitance normalized to basal cell membrane capacitance (fF/pF) in Syn-1A flox control (n = 22 cells) and Syn-1A-βKO (n = 17 cells) β-cells from three pairs of mice. Values represent mean ± S.E. (error bars); *, p < 0.05; D, size of the RRP of insulin SGs (ΔCm1st-2nd pulse) and rate of SG mobilization (ΔCm3rd-10th pulse) (n = 17–22 cells). Values represent mean ± S.E.; *, p < 0.05. E, representative traces showing Ca currents recorded in whole-cell mode from Syn-1A flox control versus Syn-1A-βKO mouse β-cells. F, current-voltage relationship of Ca channels of three pairs of Syn-1A flox control (n = 16 cells) versus Syn-1A-βKO mice β-cells (n = 20 cells) showed no significant difference. Currents were normalized to cell capacitance to yield current density.
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by a protocol consisting of a train of 10 500-ms depolarization pulses. Cell Cm changes elicited by the first two pulses have been previously postulated to approximate the size of the readily releasable pool (RRP) of primed and fusion-ready SGs (30). Subsequent pulses estimate the rate of SG refilling or mobilization from reserve pool(s) to the RRP, where SGs are subsequently primed for fusion competence (30). The size of the RRP and rate of SG mobilization are believed to correlate with first- and second-phase GSIS from pancreatic islets (30). Fig. 5, A and B, shows representative recordings of Cm from Syn-1A flox control and Syn-1A-βKO mouse β-cells. When compared with control β-cells, Cm increases in Syn-1A-βKO β-cells were reduced at every depolarizing pulse (Fig. 5C). Fig. 5D shows that the size of RRP of SGs (ΔCm1st-2nd pulse) was reduced by 59% (Syn-1A-βKO, 1.3 ± 0.2 fF/pF; Syn-1A flox control, 3.2 ± 0.6 fF/pF). As would be predicted from the EM data (Fig. 4), the rate of SG refilling/mobilization (ΔCm1st,10th pulse) was also reduced by 45% (Syn-1A-βKO, 3.9 ± 0.7 fF/pF; Syn-1A flox control, 7 ± 1.2 fF/pF).

Because Syn-1A has been shown to bind voltage-gated calcium channel (Ca$_2^+$)s to form the exocytosis complexes with cognate SNARE complex proteins in β-cells (31), we examined whether the Syn-1A deletion might cause alterations in Ca$_2^+$ currents. Between Syn-1A-βKO and control β-cells, we observed no significant changes in Ca$_2^+$ current amplitudes (Fig. 5, E and F). This suggests the possibility of redundant alternate syntaxins that might be also acting on β-cell Ca$_2^+$s (32).

**Syn-1A Deletion Reduced Fusion of Docked and Newcomer Short Dock SGs—**TIRF microscopy (6, 10, 11, 17, 18), employed to examine single insulin SG exocytotic behavior in response to physiologic glucose stimulation, has consistently shown heterogeneous SG populations, including not only pre-docked SGs but also newcomer SGs that undergo little (short dock) to no residence time (no dock) at the PM before fusion (8). Newcomer SGs account for almost all of second-phase GSIS but also a major portion of first-phase GSIS (8). At unstimulated (2.8 mmol/liter glucose) state (Fig. 6A), punctate fluorescence indicating pre-docked SGs were reduced by 66% in Syn-1A-βKO β-cells (0.059 ± 0.02) compared with Syn-1A flox control β-cells (0.173 ± 0.03), which would be consistent with the EM results (Fig. 4). When stimulated with 16.7 mmol/liter glucose, single SG fusion events observed as flashes of fluorescence that rapidly dissipate in a cloudlike diffusion pattern were in three different patterns (8). “Paddock” fusion mode (Fig. 6, B (top) and C (black)) refers to SGs already docked onto PM for a period of time before stimulation. Newcomer SGs are SGs appearing de novo after stimulation within the exocytotic field that then undergo exocytosis in two patterns. First is immediate exocytosis with a docking state of <200 ms, which is the minimal interval between two consecutive frames (Fig. 6, B (middle) and C (white)), called “no-dock” newcomer SGs. Second are SGs that dock for some residence time at the PM varying from seconds to minutes before fusion with PM (Fig. 6, B (bottom) and C (gray)), called “short-dock” newcomer SGs.

As expected, fusion of pre-docked SGs encompassing first-phase GSIS (summary analysis in Fig. 6D) was reduced by 86.9% (1.21 ± 0.21/100 μm$^2$ (Syn-1A-βKO) versus 9.25 ± 2.58/100 μm$^2$ (Syn-1A flox)) and by 90.2% in second phase (Syn-1A-βKO, 0.21 ± 0.08; control, 2.15 ± 0.65), similar to the previous report in global Syn-1A-KO mouse (6). The larger number of no-dock SGs was not significantly affected in first-phase (Fig. 6, C and D; Syn-1A-βKO, 6.22 ± 1.33; control, 7.85 ± 2.56) or second-phase GSIS (Syn-1A-βKO, 4.39 ± 1.13; control, 5.67 ± 1.08), which is also consistent with the global Syn-1A-KO β-cell report (6). Remarkably, the smaller newcomer SG population of short-dock SGs in Syn-1A-βKO β-cells were reduced by 65.9% in first phase (Syn-1A-βKO, 0.61 ± 0.35; control, 1.79 ± 0.45) and by 52.1% in second phase (Syn-1A-βKO, 1.27 ± 0.31; control, 2.65 ± 1.1) compared with Syn-1A flox control β-cells. The previous report (6) did not distinguish between the two populations of newcomer SGs. We confirmed that the deficient first-phase GSIS in Syn-1A-βKO mice islets is attributable to reduced pre-docked SGs and their fusion. However, we find that the deficient second-phase GSIS was contributed mostly from reduction in fusion of short-dock newcomer SGs. It also appears that the reduced replenishment of SGs shown in the EM analysis (Fig. 4) affected short-dock SGs more than no-dock SGs.

Syn-1A Mediates Recruitment and Fusion of Newcomer SGs Probably by Interactions with Newcomer SG VAMP8—The overriding current thinking is that Syn-1A activation by Munc18a forms a SNARE complex with VAMP2 and SNAP25 to mediate docking and fusion of pre-docked SGs (5, 6). Recently, we reported that VAMP8 is the putative VAMP mediating the recruitment and fusion of newcomer SGs (11) along with cognate t-SNARE Syn-3 (10) and that Syn-3/VAMP8/SNAP25 SNARE complex assembly could be activated by Munc18b (9). Our recent report showed that Syn-4, thought to prefer VAMP2 to mediate fusion of pre-docked SGs (15, 16), could also bind VAMP8 to mediate fusion of newcomer SGs (18). This led us to postulate that Syn-1A deletion's effects on short-dock newcomer SGs (Fig. 6) might in part be due to the promiscuous binding of Syn-1A to VAMP8. We conducted co-IP experiments with Syn-1A antibody on INS-1 cells maximally stimulated with 16.7 mmol/liter glucose plus 10 mmol/liter GLP-1 to optimally activate SM-SNARE complexes (9, 11). INS-1 was employed as surrogate for islets to provide an abundance of protein required for co-IP studies (Fig. 7). Under unstimulated conditions (0.8 mmol/liter glucose), Syn-1A co-immunoprecipitated Munc18a and minimal amounts of SNAP25 (and SNAP23) and both VAMPs, VAMP2 and VAMP8 (Fig. 7Ai; analysis of three experiments shown in Fig. 7Aii, loading controls shown in Fig. 7B). With stimulation, the amounts of SNAP25 and VAMP2 co-immunoprecipitated were increased significantly, by 336 and 161%, respectively, as expected. However, the amount of VAMP8 co-immunoprecipitated was also increased, albeit at a smaller amount of 114%. There was no change in the amounts of Munc18a or SNAP23 co-immunoprecipitated. Because binding of Syn-1A to VAMP8 in INS-1 could be attributed to other accessory proteins, we assessed their direct interactions by co-expressing Syn-1A with VAMP2-GFP or VAMP8-GFP in HEK cells and then performed IP. Syn-1A co-immunoprecipitated similar amounts of VAMP2 and VAMP8 (supplemental Fig. S3A (bottom) shows an analysis of three independent experiments). We conducted the reciprocal study of VAMP2 or VAMP8 IP with GFP anti-
body, which co-immunoprecipitated similar levels of Syn-1A (supplemental Fig. S3B (bottom) shows analysis) as well. The abundance of Syn-1A-VAMP2 complexes exceeded Syn-2-VAMP8 complexes in the more physiologic INS-1 model (Fig. 7Aii), suggesting that either other accessory proteins (i.e. Munc18s and others) in native β-cells affect SNARE complex

FIGURE 6. TIRF microscopy showing that Syn-1A deletion reduces predocked SGs in first-phase and short dock newcomer SGs in second-phase GSIS. A, left, TIRF images of docked insulin SGs in Syn-1A flox control (n = 15) versus Syn-1A-βKO mouse β-cells (n = 21) from four pairs of mice. Scale bars, 2 μm. Right, averaged SG densities on the PM before stimulation. B, kymographs and corresponding fluorescence intensity curves showing three modes of insulin SG fusion events: “predock” (black bar), “newcomer-no dock” (white bar), and “newcomer-short dock” (gray bar). C, histogram of fusion events in first (first 4 min after 16.7 mmol/liter glucose stimulation) and second phases (5–12 min) in Syn-1A flox control (n = 15 cells) versus Syn-1A-βKO mouse β-cells (n = 18 cells). D, summary of the three modes of fusion events in first (left) and second phases (right). Values are shown as mean ± S.E. (error bars). NS, not significant; *, p < 0.05; **, p < 0.005; ***, p < 0.001.
assembly, or the more rapid priming and fusion of newcomer SGs suggest faster Syn-1A/VAMP8 complex disassembly. Taken together, it appears that specific exocytotic events (pre-docked versus newcomer SGs mediating first- and second-phase GSIS) may not be dictated solely by syntaxins (Syn-1A, Syn-3, and Syn-4) but also by the v-SNAREs (VAMP2 and VAMP8) that each Syn interacts with.

Discussion
This work shows that Syn-1A mediated not only the number and fusion competence of predocked insulin SGs (6) but also the recruitment of newcomer SGs to explain the reduction of biphasic GSIS observed in vivo and in vitro from pancreatic islets of Syn-1A-βKO mice. Exocytosis of predocked insulin SGs are now known to account for only half of first-phase GSIS, whereas the other half arises from newcomer SGs, and second-phase GSIS is almost entirely attributable to newcomer SGs (8, 10, 11). It is likely that reduction of both exocytotic events would quantitatively account for the disappearance of first-phase GSIS in T2D (33), which has been attributed to 70% reduction in islet Syn-1A levels (20). Moreover, in T2D patients, second-phase GSIS is also reduced or rendered less efficient to the increasing glycemic demand (33), which probably arises from defective exocytosis of newcomer SGs. Our results suggest that this second-phase defect in T2D might also be contributed by Syn-1A deficiency. It is tempting to simply restore Syn-1A levels in the T2D islets to rescue the deficient GSIS (6). However, a previous study suggested that overexpression of Syn-1A even slightly over normal levels actually reduced GSIS (34); that was probably due to excess formation of fusion-incompetent incomplete SNARE complexes.

We partly elucidated the underlying mechanism to explain the exocytotic defects caused by Syn-1A deficiency. As expected, preferential binding of Syn-1A to VAMP2 formed the exocytotic SNARE complex with SNAP25 to mediate exocytosis of predocked SGs (3, 6). Consistently, we saw a reduced number of insulin SGs docked onto the PM, and fusion of predocked SGs was almost entirely abrogated. We had reported that Syn-3 preferentially binds VAMP8, and the Syn-3/VAMP8 SNARE complex mediated both no-dock and short-dock newcomer SGs but without any effect on pre-docked SGs (10, 11). We postulated that Syn-1A could have promiscuous binding to VAMP8 to in part explain the effects of Syn-1A-KO on short-dock newcomer SGs. Indeed, Syn-1A directly binds VAMP8, and Syn-1A/VAMP8 complex formation increased following stimulation. Thus, v-SNARE/t-SNARE pairing of Syn-1A/VAMP8 complex, like the Syn-3/VAMP8 complex, could mediate the recruitment and fusion of newcomer SGs. The binding of Syn-1A to VAMP2 and VAMP8 was mimicked by Syn-4 (18), which also affected both pre-docked and newcomer SGs. Peculiarly, Syn-4 depletion in human β-cells affected predominantly no-dock newcomer SGs (18), whereas Syn-1A-KO had preference on abrogating fusion of short-dock SGs. These studies taken together suggest that the mode of exocytosis is not solely dictated by t-SNARE syntaxins per se, but also by their respective affinity to SG v-SNAREs, VAMP2 preferring predocked SGs and VAMP8 preferring newcomer SGs (11). However, the Syn-1A/VAMP8 complex may be binding a distinct set of accessory proteins that influence docking and fusion kinetics differently from those mediated by Syn-4/VAMP8 or Syn-3/VAMP8 complexes (discussed further...
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The two last points we would like to make are with regard to the reduced Syn-1A levels found in human T2D islets (20) that underlie the rationale to pursue this study and our rationale for having used multiple strategies to elucidate the precise exocytosis steps mediated by Syn-1A in the β-cell. With regard to the first point, we also found reduced islet Syn-1A levels in two of the most frequently used T2D rodent models, the obese Zucker rat (42) and non-obese Goto-Kakizaki rat (43). Another laboratory (44) showed Syn-1A gene expression as reduced in T2D patient islets (9 T2D patients versus 55 non-diabetic donors). However, a report by Marselli et al. (45) using laser microdissection of islet cells reported that T2D human β-cells had normal Syn-1A levels.

In this study, we have opted to employ multiple strategies to take advantage of the major strength of each method and offset the inherent weakness of another; thus, these methodologies would be complementary, and results would be mutually confirmatory and therefore more unequivocal. The often used patch clamp depolarization-induced exocytosis assay does not mimic glucose-mediated exocytosis. Initial depolarizations (first two pulses) would release the pre-docked SGs located in close vicinity of the Ca²⁺ channels first (designated as the RRP), and the larger Ca²⁺ influx from subsequent depolarization pulses would diffuse further into the cytoplasm to mobilize and induce fusion of SGs located further into the cell interior (30), which would cause fusion of predominantly newcomer SGs. Although patch clamp protocols were designed to select out the fusion of pre-docked SGs within the RRP in a manner “disrupted” from newcomer SGs, which has been a widely accepted assumption, this may no longer be the case. We have reported in our VAMP8 KO mouse β-cell study (11) that VAMP8 mediated only newcomer SG fusion but appeared to contribute substantially to the RRP using patch clamp capacitance measurements. Thus, to unequivocally distinguish between the population of pre-docked and newcomer SG fusion and in response to physiologic stimulus glucose, we have preferred the TIRF microscopy approach to unequivocally show these single SG events at the highest spatio-temporal resolution. Nonetheless, TIRF microscopy is limited to visualizing the events at or close to the PM and could not unequivocally assess the mobilization of SGs per se from the cell interior. Strategies to assess SG mobilization and replenishment from the cell interior are very limited, and we believe the strongest one would be by EM fusion defect at the PM, then SGs will accumulate near the PM. Therefore, Syn-1A deletion caused a mobilization defect in the SGs at least in the context of the islet β-cell, which is a novel finding and a new secretory function for Syn-1A.

**Experimental Procedures**

**Generation of β-Cell-specific Syn-1A-KO Mice**—We used conventional embryonic stem (ES) cell targeting to generate pluripotent ES cells containing a floxed allele of Syn-1A.
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homologous recombination with pNeoLoxPTK targeting vector, loxp sites were inserted to flank exons 2 and 3 of Syn-1A (Fig. 1A). Correct targeting was confirmed by Southern blotting analysis, and chimeras were generated by aggregating four independent ES cell clones with 8-cell stage embryos. Male chimeras were used for germ line transmission breeding. Mice expressing Cre recombinase under the control of a promoter that drives expression only in islet β-cells, transgene mice Tg(Ins2-Cre)Herr called RIP-Cre mice, here were used for breeding with Syn-1A floxed mice to generate β-cell-specific KO of Syn-1A (Syn-1A−/−KO). 12–14-week-old Syn-1A floxed and Tg(Ins2-Cre)Herr mice were backcrossed to C57BL/6J mice for eight generations before offspring were used for experiments. All procedures were in accordance with Canadian Council on Animal Care Standards and approved by the University of Toronto Animal Care Committee.

Confocal Microscopy—Mouse islets were fixed in 4% paraformaldehyde (45 min), permeabilized with 0.1% Triton X-100 (45 min), blocked with 10% normal goat serum (1 h), and then stained with primary antibodies (1:200 dilution, 3 h) (mouse monoclonal antibodies against insulin (Sigma-Aldrich), glucagon (Sigma-Aldrich), and somatostatin (GeneTex, Irvine, CA) or rabbit anti-syntaxin-1A (Synaptic Systems, Goettingen, Germany)) and then tagged with anti-mouse IgG FITC or anti-rabbit IgG Texas Red secondary antibody (AbD Serotec, Raleigh, NC; 1:1000 dilution, 2 h). Stained islets were imaged under a spinning disc confocal microscope (Leica Microsystems, Wetzlar, Germany), and data were acquired and analyzed by Velocity software (PerkinElmer Life Sciences).

IPGTT—IPGTTs (2 g of glucose/kg body weight) on 12-week-old male mice were performed after an 18-h fast. Blood samples were then collected from the tail vein without anesthesia. Insulin levels were determined by a radioimmune assay (EMD Millipore Corp., Bilicera, MA).

Insulin Secretion—Perifusion assays on mouse pancreatic islets (70 islets/chamber) were performed to assess biphasic GSIS as reported (9, 11). Insulin secreted was determined by a radioimmune assay.

Electron Microscopy—As described previously (10, 11), mouse islets were fixed for 1 h with Karnovsky style fixative (3.2% paraformaldehyde, 2.5% glutaraldehyde) in 0.1 mol/liter sodium cacodylate buffer (pH 6.5) with 5 mmol/liter CaCl$_2$$_2$, postfixed for 30 min with 1% osmium tetroxide. Samples were embedded in 1% uranyl acetate (1 h), dehydrated, and infiltrated with Epon 812 resin. Polymerization was completed by epoxy resin, forming a solid epoxy disk, which was subjected to ultrathin sectioning (80 nm) using a Reichert Ultracut E5 microtome. Slices were collected on 200-mesh copper grids, counterstained (15 min) using saturated 4% uranyl acetate followed by Reynold’s lead citrate, and then examined in a Hitachi H-7000 transmission electron microscope (Krefeld, Germany) at an accelerating voltage of 75 kV and photographed with an AMT XR-60 camera and software. For morphometric analysis (ImageJ), concentric shells of 0.2 μm from the PM to the cell interior up to 1.5 μm were drawn, and then the centers of SGs within the shell were determined and counted.

Electrophysiology—This was performed as described (11, 41). Cells were patch-clamped in conventional whole-cell configuration. Cm (11) and Ca$_v$ current measurements (41) were performed using an EPC-10 amplifier and PULSE and X-Chart software (HEKA Electronik, Lambrecht, Germany). Patch clamp data were analyzed with Igor Pro software (WaveMetrics, Portland, OR). For measurement of Ca$_v$ currents (41), barium was used as a charge carrier, and pipettes were filled with 120 mmol/liter CsCl, 20 mmol/liter tetraethylammonium Cl, 5 mmol/liter EGTA, 5 mmol/liter MgATP, 5 mmol/liter HEPES (pH 7.2), whereas external solution contained 100 mmol/liter NaCl, 20 mmol/liter BaCl$_2$, 20 mmol/liter tetraethylammonium Cl, 4 mmol/liter CsCl, 1 mmol/liter MgCl$_2$, 10 mmol/liter glucose, 5 mmol/liter HEPES (pH 7.4). Cells were held at −70 mV for 2 min after the formation of whole-cell mode, and currents were elicited by steps of 500-ms depolarizations from −80 to +60 mV in 10-mV increments.

TIRF Imaging—TIRF microscopy was performed as reported (9–11) using a Nikon TE2000U TIRF microscope (Nikon Canada, Mississauga, Canada), with images obtained at 5 Hz, 100-ms exposure time. Large round cells were chosen as β-cells, which were confirmed by their response to high glucose stimulation. Fusion events observed as flashes of fluorescence indicating emptying of NPY-EGFP cargo (mouse β-cells infected with Ad-NPY-EGFP) were manually selected. Two concentric circles (5 and 7 pixels with a pixel size of 267 nm, corresponding to 1.3- and 1.8-μm diameter) were used to center on selected SGs. Evolution of fluorescence changes over time of single SGs was analyzed by Matlab (Math-Works, Natick, MA), ImageJ (National Institutes of Health, Bethesda, MD), and Igor Pro software (WaveMetrics), whereby dissipation of average fluorescence in the concentric annulus was considered to be release of SG cargo. Before image acquisition, cells were preincubated for 30 min in KRB buffer containing 2.8 mmol/liter glucose and then stimulated with 16.7 mmol/liter glucose for 12 min.

Co-immunoprecipitation and Western Blotting—Western blots of islets, INS-1, or HEK cell lysate samples were performed with the indicated antibodies: VAMP2 (1:2000; Anson Lowe (Stanford University)); VAMP8 (1:1000; C.C. Wang and W.J. Hong (Institute of Molecular and Cell Biology, Singapore)); Syn-1A (1:1000), tubulin (1:1000) (Sigma-Aldrich); Syn-2 (1:1200), Syn-3 (1:1000), and SNAP-23 (1:1000) (Synaptic Systems); SNAP-25 (1:1500; Sternberger Monoclonal, Covance, NJ); Munc18a (1:1000; BD Biosciences); Munc18b (1:1000; V. Olkkonen (Minerva Foundation Institute, Helsinki, Finland)); and GFP (1:1000; Clontech). The noncommercial antibodies for VAMP2, VAMP8, and Munc18b were validated in our previous papers (9–11). Protein bands were visualized by chemiluminescence (Pierce, Nepean, Canada).

INS-1 cells (70–75% confluence) were treated as indicated; then 1 mg of protein of INS-1 lysate was subjected to immunoprecipitation with Syn-1A antibody (2 μg) cross-linked to protein A-agarose beads (Molecular Probes, Inc., Eugene, OR) performed as described previously (9, 11). Co-precipitated proteins were identified by Western blotting. For accurate comparison, VAMP2 and VAMP8 (also SNAP25 and SNAP23) were probed simultaneously and separated well enough on PAGE.

HEK cells were transfected with Syn-1A and then infected with Ad-VAMP2-EGFP or Ad-VAMP8-EGFP virus. 2 days
after infection, cells were collected for IP. Cell lysates were prepared by using the above procedure and then incubated with protein A-agarose-cross-linked anti-Syn-1A or anti-GFP antibody. Precipitated proteins were washed with IP buffer three times, separated on 15% SDS-PAGE, and identified with anti-GFP or anti-Syn-1A antibody.

**Gene Expression**—This was performed as described previously (46). Total RNA was extracted from mouse hypothalamus using the Qiagen RNAeasy Plus minikit (Hilden, Germany). 1–5 μg of isolated RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Sigma-Aldrich). Real-time PCR was performed in a 384-well plate with 5 μl of SYBR Green PCR Master Mix (ThermoFisher, Waltham, MA), 0.8 μl of ultrapure water, 0.2 μl of 50 μmol/liter forward and reverse primer mix (primer sequence was shown in supplemental Table S1), and 4 μl of sample containing 10 ng of cDNA/well. The real-time PCR protocol employed was as follows: heat activation of polymerase at 95 °C for 3 min, followed by 40 cycles of the following: 95 °C for 10 s, 65 °C for 15 s, and 72 °C for 20 s. Readings were carried out on a VIIA7 real-time PCR system (ThermoFisher) and compared against a standard curve created from mouse genomic DNA. Data were normalized to expression of β-actin.

**Statistical Analysis**—All data are presented as mean ± S.E. Statistical significance was evaluated by a two-tailed t test using SigmaStat (Systat Software, Chicago, IL). Significant difference is indicated by asterisks (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

**Author Contributions**—T. L. and T. Q. performed most of the experiments, their design, and data analysis. L. X., S. D., D. Z., Y. K., and K. J. P. contributed to some experiments. L. O. made the KO mouse. T. L. and T. Q. performed most of the experiments, their design, and data analysis. L. X., S. D., D. Z., Y. K., and K. J. P. contributed to some experiments. L. O. made the KO mouse. T. L. and T. Q. performed most of the experiments, their design, and data analysis. L. X., S. D., D. Z., Y. K., and K. J. P. contributed to some experiments. L. O. made the KO mouse.

**Acknowledgments**—Some equipment used in this study was supported by the 3D (Diet, Digestive Tract, and Disease) Centre funded by the Canadian Foundation for Innovation and Ontario Research Fund, project number 19442, and the Banting and Best Diabetes Center of the University of Toronto.

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