Supplemental methods and data

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PS labeling and confocal microscopy

To test labeling of PS in the outer leaflet of PC12 cell plasma membranes, 10 µl annexin-V-FITC (SouthernBiotech, Birmingham, AL) was added to intact cells incubated in annexin-binding-buffer (10 mM HEPES, 140 mM NaCl, 3 mM $CaCl_2$, pH 7.4) and the cells were visualized after 1 min. To label PS after flipping to the extracellular surface of the plasma membrane, cells were bathed in medium containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, 2 mM $CaCl_2$, 10 µM ionomycin for 5 min followed by addition of 10 µl annexin-V-FITC. Cells were visualized after 1 min. To stain intracellular PS, cells were fixed with 4% paraformaldehyde for 1 hr and then washed 3 times in PBS. The plasma membrane was permeabilized using 0.3% Triton X-100 and washed 3x in PBS. Cells were incubated with 10 µl annexin-V-FITC in 500 µl annexin-binding-buffer with 3 mM $CaCl_2$. After treatment, cells were viewed at room temperature (~22 ^{o}C) with a confocal microscope (Fluoview FV1000; Olympus, Center Valley, PA) using a 60X 1.2 NA objective. Images were acquired and analyzed with Fluoview software (Olympus). Sample cells were cropped to prepare the figure.

Ca^{2+} current

Ca^{2+} current was measured in whole-cell patch-clamp recordings from cells bathing in 105 mM NaCl, 20 mM $CaCl_2$, 1.5 mM $MgSO_4$, 15 mM glucose, 0.3 µM tetrodotoxin, and 5 mM HEPES, pH 7.35. Patch pipettes were filled with 90 mM N-methylglucamine, 20 mM TEA, 10 mM EGTA, 2 mM $MgCl_2$, 20 mM Na_2-creatine phosphate, 5 mM Mg-ATP, 0.1 mM GTP, and 10 mM HEPES, pH 7.35. Cells were held at -80 mV, and stepped for 200 msec to levels ranging from -50 to +60 mV (in 10 mV increments).

Protein Analysis in PC12 Cells

PC12 cells were harvested in PBS with 1% Triton X-100 and 5 mM PMSF. Samples were incubated at 4 °C for 30 min and then centrifuged at 21,000 g for 10 min. Supernatants were transferred to new tubes and protein concentration was determined using the BCA protein assay kit (Pierce Chemical, Rockford IL). Sample volumes were adjusted to have the same protein concentration and subjected to SDS-PAGE. Proteins were detected with monoclonal antibodies against synaptobrevin (Cl 69.1), SNAP-25 (Cl 71.1), syntaxin 1 (HPC-1), and synaptotagmin 1 (Cl 41.1), followed by secondary horse radish peroxidase conjuagated antibody, and development with enhanced chemiluminescence (Pierce Chemical).

Electron Microscopy

Cells (control, PSS2-R97K, and PSS2-R97K+PS) were fixed for 2 h with 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, rinsed three times with this buffer, and dehydrated in a graded ethanol series to 100%. Samples were infiltrated with LR White and embedded in gelatin. The embedding medium was polymerized for 48 h at 60 °C. Areas of interest were cut out and sectioned to coated slot grids. The sections were viewed with a Philips CM120 electron microscope. Image J software was used to measure diameters of vesicles.

Figure Legends

Fig S1. Analysis of PC12 cell phospholipids. (a) HPLC of total lipids provides a measure of percent PS, along with phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), and diphosphatidylglycerol (DPG = cardiolipin). A normal-phase (silica column) HPLC trace of total lipids extracted from PC12 cells shows the major phospholipid peaks, detected by absorbance at 206 nm. (b) Plot of PC content versus PS content. (c) Plot of PE content versus PS content. (d) PS content of the lipids isolated from plasma membrane plotted versus PS content of total cell lipids. (e) PS in the inner leaflet of the plasma membrane. Annexin-V-FITC specifically binds PS. Cells (control, PSS2-R97K, and PSS2-R97K+PS) were incubated for 1 min with a solution containing annexin-V-FITC. The first row shows

intact cells; no fluorescence was seen. In the second row cells were first incubated with ionomycin, which activates scramblase (Zwaal *et al.*, 2005) and flips PS from the inner leaflet to the outer leaflet. These cells exhibited annexin-V-FITC staining, with the brightest fluorescence in PSS2-R97K+PS cells. In the third row, cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and labeled with annexin-V-FITC. Again, PSS2-R97K+PS cells showed the brightest fluorescence. Error bars in b and c represent S.E.M.

Fig. S2. PS does not alter spike shape. Spike peak height, rise time, half width, decay time, total area, and PSF amplitude were measured from amperometry recordings made from PC12 cells, as described previously (Zhang and Jackson, 2008) and plotted versus PS content for each of the different conditions of elevated PS content. All plots showed no significant dependence on PS content.

Fig S3. Ca^{2+} currents were indistinguishable in PC12 cells with different PS content. (A) Representative Ca^{2+} current traces evoked by depolarizing steps to different voltages from a control PC12 cell. (B) Plot of peak Ca^{2+} current versus voltage for control, PSS2-R97K, and PSS2-R97K+PS PC12 cells. (C) Peak currents from PC12 cells under various conditions were indistinguishable. Error bars represent S.E.M. 7-10 cells were used for each condition.

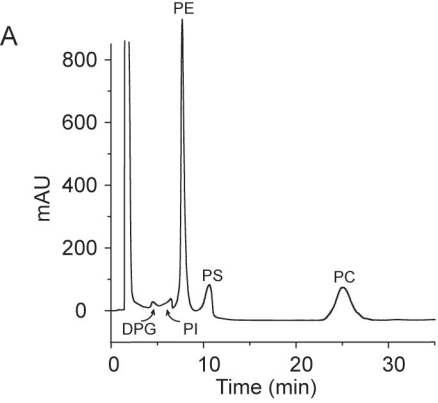
Fig S4. PS does not alter vesicle size. Electron microscope images for control (A), PSS2-R97K (B), and PSS2-R97K+PS (C). Scale bar is 1 μm for large images and 200 nm for insets. (D) Mean vesicle diameters and vesicle number per cell were indistinguishable for all three conditions. Error bars represent S.E.M. 421-724 vesicles were measured from 62-77 images to calculate vesicle seminars. Ten cells were measured to calculate vesicle numbers per cell for each condition.

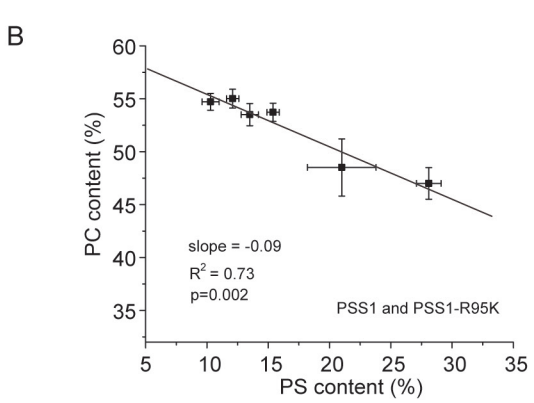
Fig. S5. A. Western blots of synaptobrevin, SNAP-25, Syntaxin 1A, Syntaxin 1B, and Synaptotagmin I in control PC12 cells, PSS2-R97K PC12 cells, and PSS2-R97K PC12 cells with added PS. **B**. Densitometry of western blots from **A**, analyzed with the program Image J (N=3). This experiment showed that raising PS to the levels indicated in Table 1 did not alter the expression of these exocytosis proteins.

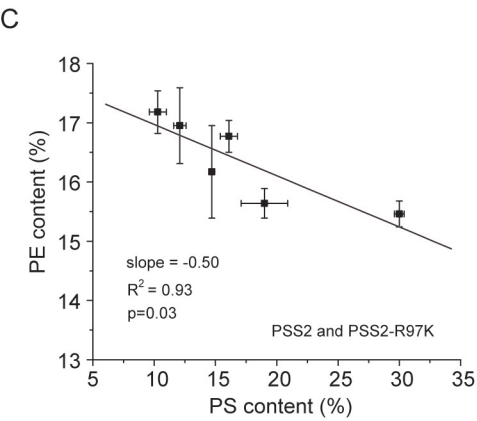
References

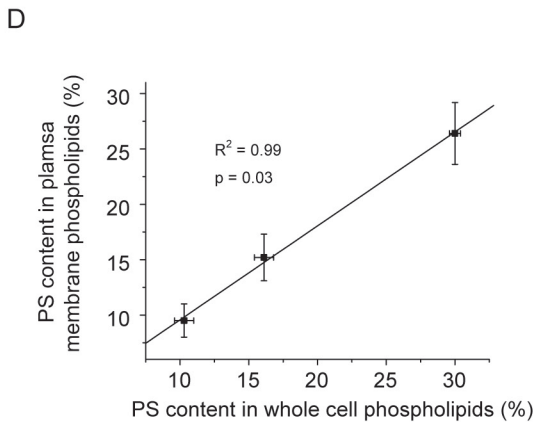
Zhang, Z., and Jackson, M.B. (2008). Temperature dependence of fusion kinetics and fusion pores in Ca2+-triggered exocytosis from PC12 cells. J Gen Physiol *131*, 117-124.

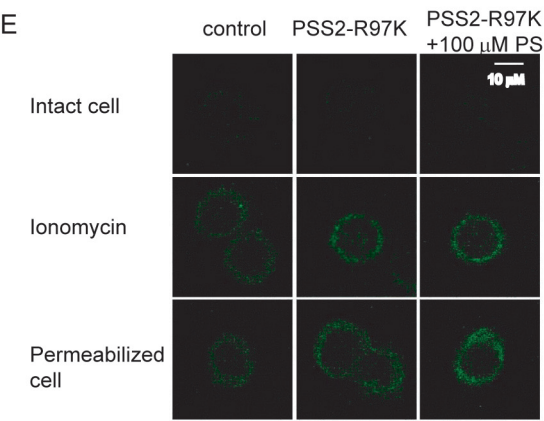
Zwaal, R.F., Comfurius, P., and Bevers, E.M. (2005). Surface exposure of phosphatidylserine in pathological cells. Cell Mol Life Sci *62*, 971-988.

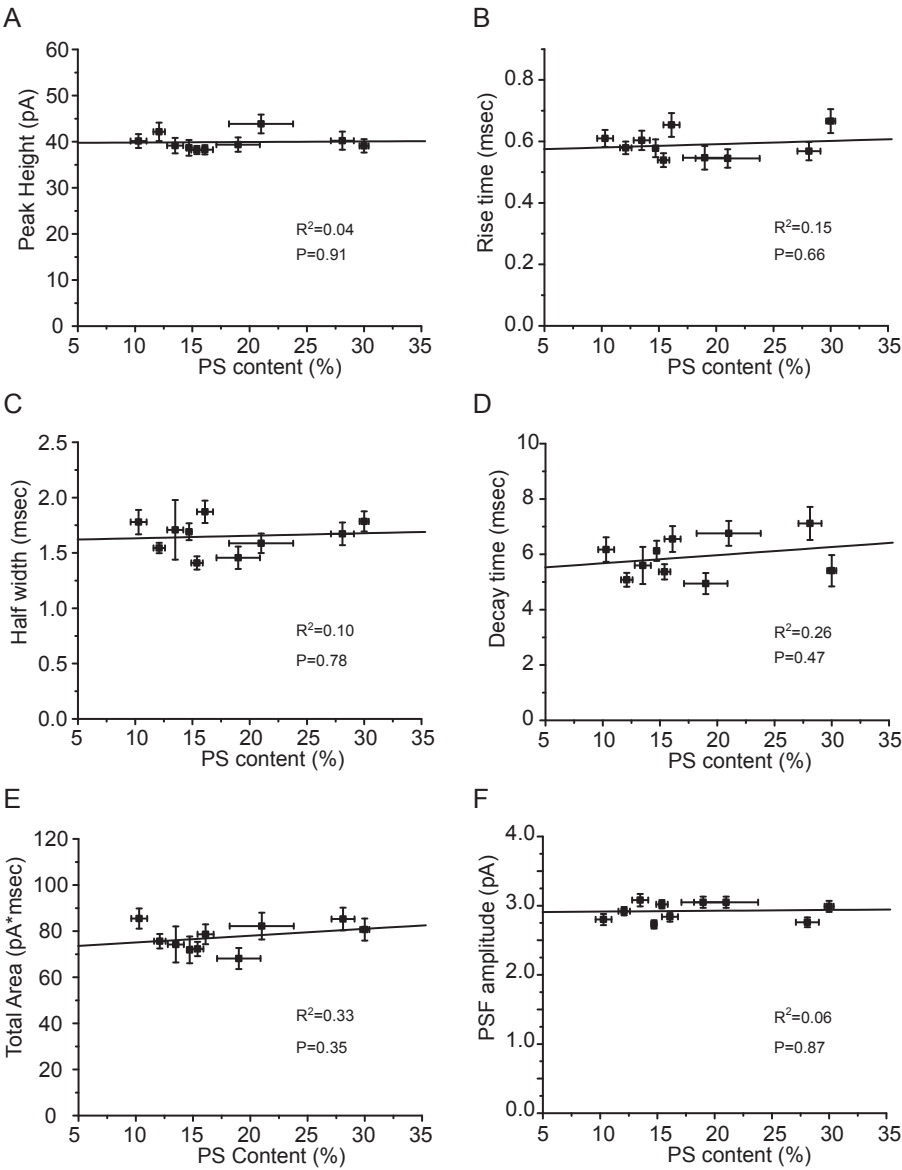

A
PE
800
600
400
200
0
mAU
PS
PC
DPG
PI
0
10
20
30
Time (min)

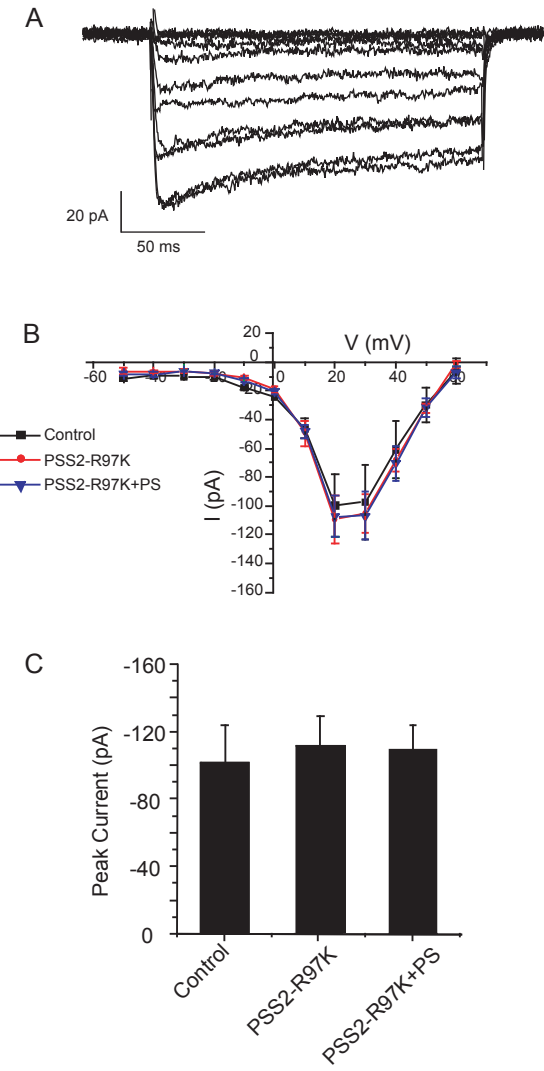

B
60
55
50
45
40
35
PC content (%)
slope = -0.09
R2 = 0.73
p=0.002
PSS1 and PSS1-R95K
5
10
15
20
25
30
35
PS content (%)

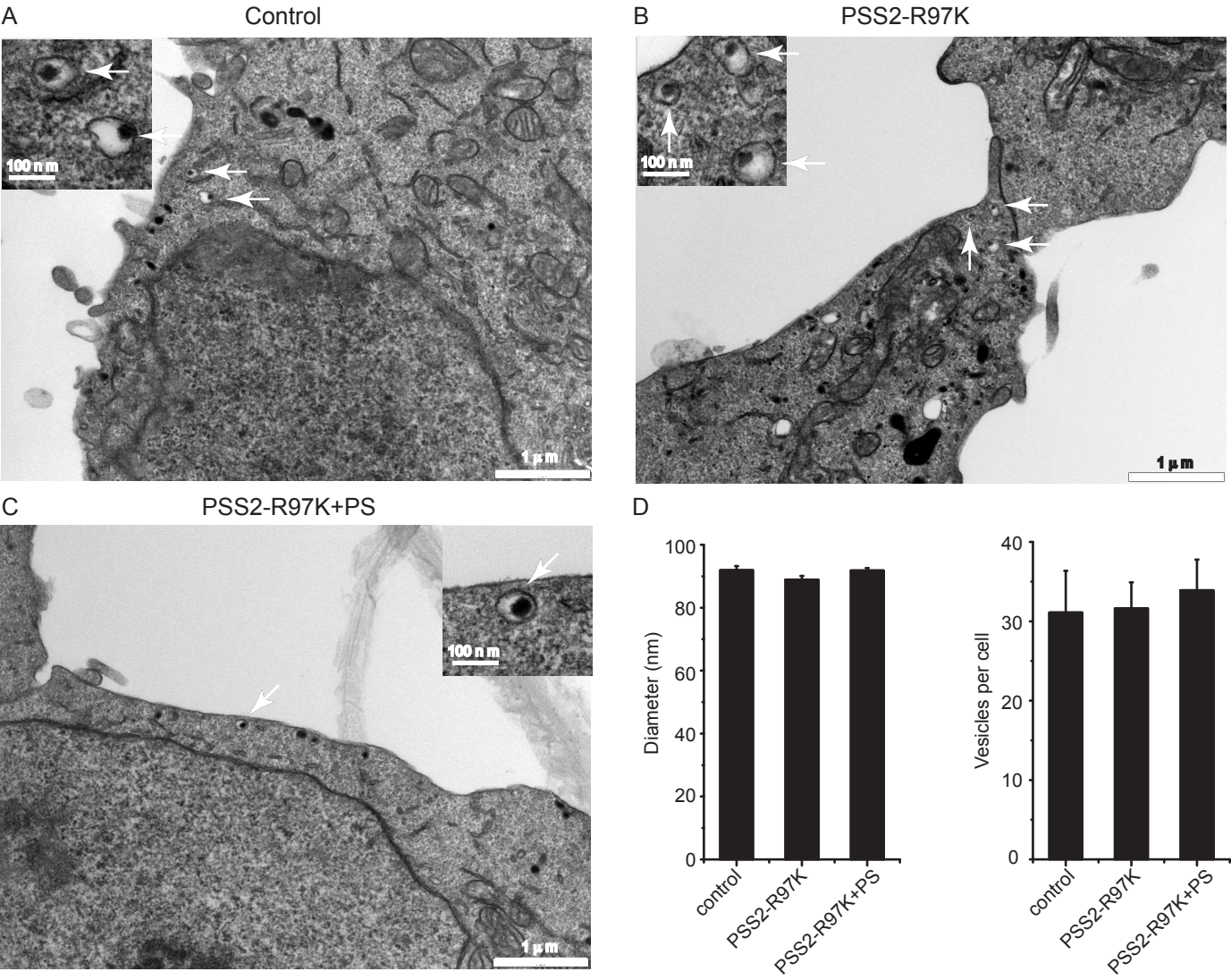

C
18
17
16
15
14
13
PE content (%)
slope = -0.50
R2 = 0.93
p=0.03
PSS2 and PSS2-R97K
5
10
15
20
25
30
35
PS content (%)

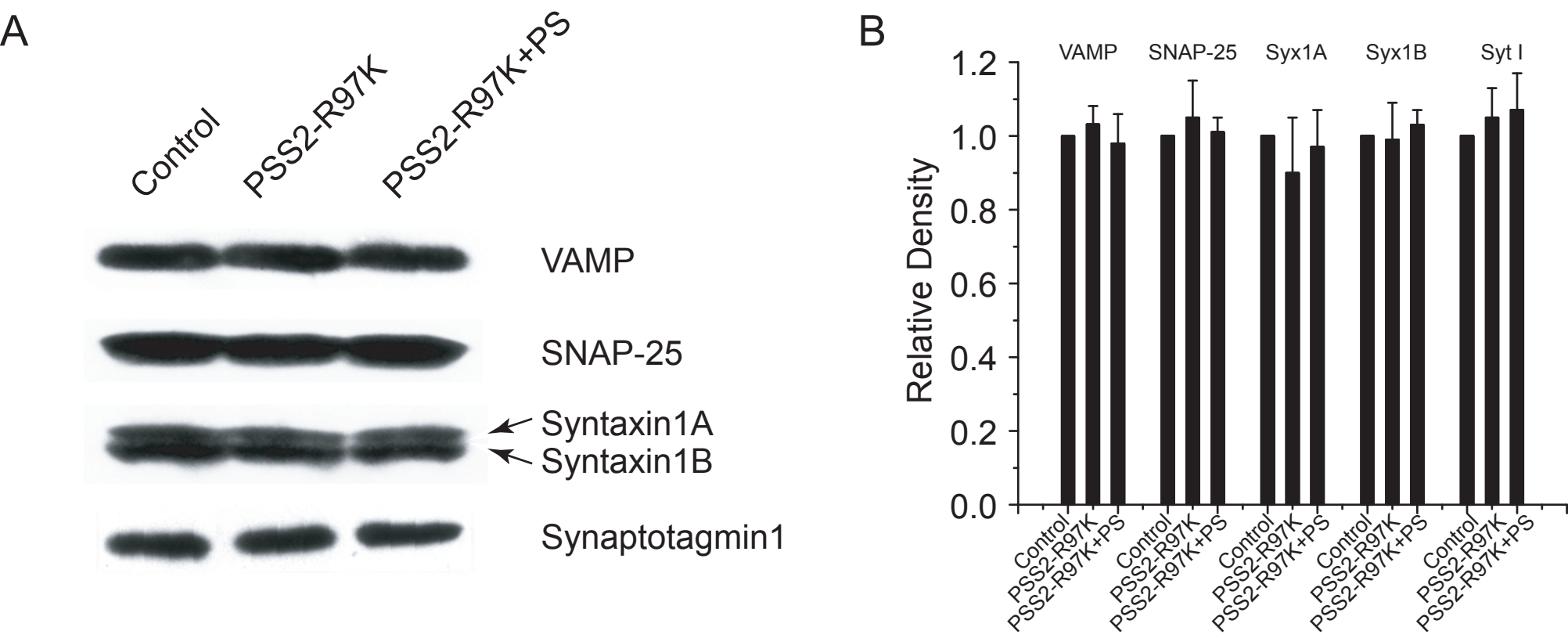

D
30
25
20
15
10
PS content in plamsa membrane phospholipids (%)
R2 = 0.99
p = 0.03
10
15
20
25
30
PS content in whole cell phospholipids (%)


E
control
PSS2-R97K
PSS2-R97K
+100 μM PS
10 μM
Intact cell
Ionomycin
Permeabilized cell


A
Peak Height (pA)
PS content (%)
R2=0.04
P=0.91
B
Rise time (msec)
PS content (%)
R2=0.15
P=0.66
C
Half width (msec)
PS content (%)
R2=0.10
P=0.78
D
Decay time (msec)
PS content (%)
R2=0.26
P=0.47
E
Total Area (pA*msec)
PS Content (%)
R2=0.33
P=0.35
F
PSF amplitude (pA)
PS content (%)
R2=0.06
P=0.87


A
20 pA
50 ms
B
V (mV)
I (pA)
Control
PSS2-R97K
PSS2-R97K+PS
C
Peak Current (pA)
Control
PSS2-R97K
PSS2-R97K+PS


A
Control
100 nm
1 μm
B
PSS2-R97K
100 nm
1 μm
C
PSS2-R97K+PS
100 nm
1 μm
D
Diameter (nm)
100
80
60
40
20
0
control
PSS2-R97K
PSS2-R97K+PS
Vesicles per cell
40
30
20
10
0
control
PSS2-R97K
PSS2-R97K+PS


A
Control
PSS2-R97K
PSS2-R97K+PS
VAMP
SNAP-25
Syntaxin1A
Syntaxin1B
Synaptotagmin1
B
VAMP
SNAP-25
Syx1A
Syx1B
Syt I
Relative Density
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
PSS2-R97K
PSS2-R97K+PS