Synaptotagmin VII Is Targeted to Dense-core Vesicles and Regulates Their Ca\(^{2+}\)-dependent Exocytosis in PC12 Cells*§

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It has recently been proposed that synaptotagmin (Syt) VII functions as a plasma membrane Ca\(^{2+}\) sensor for dense-core vesicle exocytosis in PC12 cells based on the results of transient overexpression studies using green fluorescent protein (GFP)-tagged Syt VII; however, the precise subcellular localization of Syt VII is still a matter of controversy (plasma membrane versus lysosomes). In this study we established a PC12 cell line “stably expressing” the Syt VII-GFP molecule and demonstrated by immunocytochemical and immunoelectron microscopic analyses that the Syt VII-GFP protein is localized on dense-core vesicles as well as in other intracellular membranous structures, such as the trans-Golgi network and lysosomes. Syt VII-GFP forms a complex with endogenous Syts I and IX, but not with Syt IV, and it colocalize well with Syts I and IX in the cellular processes (where dense-core vesicles are accumulated) in the PC12 cell line. We further demonstrated by an N-terminal antibody-uptake experiment that Syt VII-GFP-containing dense-core vesicles undergo Ca\(^{2+}\)-dependent exocytosis, the same as endogenous Syt IX-containing vesicles. Moreover, silencing of Syt VII-GFP with specific small interfering RNA dramatically reduced high KCl-dependent neuropeptide Y secretion from the stable PC12 cell line (~60% of the control cells), whereas the same small interfering RNA had little effect on neuropeptide Y secretion from the wild-type PC12 cells (~85–90% of the control cells), indicating that the level of endogenous expression of Syt VII molecules must be low. Our results indicate that the targeting of Syt VII-GFP molecules to specific membrane compartment(s) is affected by the transfection method (transient expression versus stable expression) and suggested that Syt VII molecule on dense-core vesicles functions as a vesicular Ca\(^{2+}\) sensor for exocytosis in endocrine cells.

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The abbreviations used are: Syts(s), synaptotagmin(s); GFP, green fluorescence protein; GST, glutathione S-transferase; NPY, neuropeptide Y; RNAi, double-stranded RNA-mediated interference; siRNA, small interfering RNA; TGN, trans-Golgi network; SNARe, soluble NSF attachment protein receptors.

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technology (reviewed in Refs. 46 and 47) causes inhibition of dense-core vesicle exocytosis in the stable PC12 cell line. Based on our findings, we propose that the Syt VII molecule is mainly targeted to dense-core vesicles and functions as a "vesicular Ca$^{2+}$" sensor in endocrine cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-Rab3A, anti-Syt IX (previously called Syt V) (8), anti-TGN38, anti-EEA1, and anti-GM130 mouse monoclonal antibodies were obtained from BD Transduction Laboratories. Anti-Syt I (SYA148) and anti-BiP monoclonal antibodies were purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia). Anti-Herpes receptor mouse monoclonal antibody and anti-Syt VII rabbit polyclonal antibody (38) were from Chemicon International Inc. (Temecula, CA) and Synaptic Systems (Göttingen, Germany), respectively. Anti-FLAG M2 mouse monoclonal antibody and anti-FLAG M2 affinity gel were from Sigma Chemical Co. Alexa Fluor 568-labeled anti-mouse IgG, Alexa Fluor 568-labeled anti-rabbit IgG, Alexa Fluor 633-labeled anti-rabbit IgG, and dextran, Texas Red, 10,000 MW, lysine-fixable were obtained from Molecular Probes Inc. (Eugene, OR). β-Nerve growth factor was from Calbiochem-Novabiochem Corp. Anti-Syt I, anti-Syt IV, anti-Syt IX-N rabbit polyclonal antibodies were prepared as described previously (25, 48–50).

**Cell Culture, Immunocytochemistry, and Immunoelectron Microscopy**—PC12 cell lines stably expressing FLAG-Syt VII-GFP (Syt VII and GFP were separated by a Gly linker) were established as described elsewhere (40, 45) (see Fig. 2A). The cells were cultured on glass bottom dishes (35-mm dish, MatTek Corp., Ashland, MA) coated with collagen type IV as described previously (49, 51). The cells were treated with 100 ng/ml β-nerve growth factor for 2 days as described previously (49). To label lysosomes in vivo, the cells were incubated with 0.5 mg/ml Texas Red-labeled dextran (10,000 MW) in culture medium for 3 h. After the cells were washed extensively with phosphate-buffered saline, they were further incubated with culture medium without dextran for 3 h as described previously (32). The cells were then fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 20 min at room temperature. Cells were then washed five times, with 0.3% Triton X-100 in phosphate-buffered saline for 2 min, and incubated with the blocking solution (1% bovine serum albumin and 0.1% Triton X-100 in phosphate-buffered saline) for 1 h at room temperature as described previously (49, 51). Next, the cells were incubated with one of the following primary antibodies, anti-Syt IV rabbit polyclonal antibody (5 µg/ml), anti-Syt I (1/250 dilution), anti-Syt IX (1/100 dilution), anti-Rab3A (1/500 dilution), anti-Transferrin receptor (1/100 dilution), anti-GM130 (1/100 dilution), anti-TGN38 (1/500 dilution), anti-BiP (1/200 dilution), or anti-EEA1 mouse monoclonal antibody (1/100 dilution). After washing the cells with the blocking solution, they were incubated with the following secondary antibodies, Alexa Fluor 568-labeled anti-rabbit IgG (1/5000 dilution) or Alexa Fluor 633-labeled anti-mouse IgG (1/5000 dilution) for 1 h at room temperature. The cells were then examined with a confocal fluorescence microscope (Fluoview, Olympus, Tokyo, Japan), and the images were processed with Adobe Photoshop software (version 7.0). The immunoelectron microscopic analysis of Syt VII-GFP molecule was essentially performed with anti-GFP rabbit polyclonal antibody (Clontech Laboratories, Inc.) as described previously (50, 52).

**Antibody-uptake Experiments**—PC12 cells cultured on 35-mm glass bottom dishes were incubated for 15 min at 37 °C with the anti-Syt IX-N rabbit polyclonal antibody (10 µg/ml) and anti-FLAG tag mouse monoclonal antibody (1 µg/ml) in either high KCl buffer (56 mM KCl, 95 mM NaCl, 2.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, and 15 mM HEPES-KOH, pH 7.4) or low KCl buffer (5.6 mM KCl, 145 mM NaCl, 2.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, and 15 mM HEPES-KOH, pH 7.4) as described previously (25, 45, 50). The cells were immediately washed twice with phosphate-buffered saline and then fixed with 4% paraformaldehyde as described above. Incorporated anti-FLAG tag mouse antibody and anti-Syt IX-N rabbit antibody were separately visualized with Alexa Fluor 568-labeled anti-rabbit and Alexa Fluor 633-labeled anti-rabbit IgGs (1/5000 dilution), respectively, and then analyzed with a confocal fluorescence microscope as described above.

**Immunoblotting and Immunoprecipitation**—FLAG-Syt VII-GFP-expressing PC12 cells (two confluent 10-cm dishes) were solubilized in a buffer containing 1 µl of 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, and 10 µM pepstatin A) in a glass-Teflon Potter homogenizer with 10 strokes at 900–1000 rpm, and the proteins were solubilized with 1% Triton X-100 at 4 °C for 1 h. After removing the insoluble material by centrifugation at 15,000 rpm for 10 min, the supernatant (400 µl) was incubated with anti-FLAG M2 affinity gel in the presence of 1 mM CaCl$_2$ (or 2 mM EGTA) for 1 h at 4 °C. The beads were then washed five times with 10 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 0.2% Triton X-100, 1 mM CaCl$_2$ (or 2 mM EGTA), and protease inhibitors, and the immunoprecipitates were subjected to a 10% SDS-PAGE followed by immunoblotting with anti-Syt I (1/250 dilution), anti-Syt IV (0.9 µg/ml), anti-Syt IX (1/250 dilution), or horseradish peroxidase-conjugated anti-FLAG tag mouse monoclonal antibody (1/10,000 dilution) as described previously (53). Immunoreactive bands were visualized with enhanced chemiluminescence (Amersham Biosciences). The blots shown in this paper are representative of three independent experiments.

**Construction of pSilencer-Syt VII Vectors for Syt VII Silencing and Neuropeptide Y (NPY) Release Assay**—To silence rat and mouse Syt VII, a 19-nucleotide sequence (TCATCACCGTCAGCCTTAG) was selected according to the manufacturer’s notes (Ambion, Inc., Austin, TX). The specificity of the sequence was verified by a BLAST search of the public data bases. Silencer™ 1.0-U6 expression vectors (Ambion) that produce small interfering RNAs (siRNA) targeted against Syt VII (named pSilencer-Syt VII) were also prepared according to the manufacturer’s notes (29). In brief, two oligonucleotides (sense, 5'-TCATCACCGTGACCTTGTCAAGAAGATGAGGATCGTGTAGATGTATTTTT-3' and its complement) were annealed and ligated into the pSilencer vector, followed by transfection into PC12 cells with transfection reagent

**Fig. 1.** Colocalization of Syt VII-GFP with dense-core vesicle markers (Syt I, Syt IX, and Rab3A) in the PC12 cell line. Syt VII-GFP-expressing PC12 cells (A, D, G, and J, green) were fixed, permeabilized, and stained with anti-Syt I (B, red), anti-Syt IX (E, red), anti-Syt IV (H, red), or anti-Rab3A (K, red). C, F, I, and L are superpositions of A and B, D and E, G and H, and J and K, respectively. Note that the Syt VII-GFP signals at the cell periphery colocalized well with the dense-core vesicle markers (Syt I, Syt IX, and Rab3A; yellow in C, F, and L, respectively), especially at the tips of the cellular processes, consistent with the results of our previous subcellular fractionation study (40). Because the Syt IV protein in undifferentiated PC12 cells is mainly localized in the Golgi and immature secretory vesicles (49, 50, 67), no colocalization between Syt VII-GFP and Syt IV was observed even in the tips of the cellular processes (I, inset, arrow) or in the perinuclear region. Confocal images were taken to highlight the localization of Syt VII-GFP in the tips of the cellular processes (i.e. dense-core vesicle markers are accumulated), and as a result perinuclear localization of Syt VII-GFP is not so evident in Fig. 1 (see Fig. 4). Scale bar = 20 µm.
tandem C2 domains (the coimmunoprecipitated with Syt VII-GFP irrespective of the presence of Ca\(^{2+}\)).

The NPY cDNA was provided by Dr. Wolfhard Almers (Portland, OR) (56). We then selected three independent cell lines and found that they exhibited the same intracellular distribution of Syt VII-GFP (Figs. 1 and 4, A). Thus, transiently overexpressed Syt VII molecules, it is highly possible that excess recombinant Syt VII protein is mistargeted to other intracellular compartments by overflowing the normal transport pathway. To investigate this possibility, we established a PC12 cell line that stably expresses FLAG-Syt VII-GFP molecule (29, 54) (see Fig. 6 A).

Cotransfection of pShooter-NPY-T7-GST with pSilencer-Syt VII into PC12 cells and measurement of the NPY released with high (or low) Ca\(^{2+}\) was performed using anti-FLAG M2 affinity gel beads in the presence or absence of 1 mM Ca\(^{2+}\) (indicated as + or −, respectively) as described under “Experimental Procedures.” Proteins trapped by the beads were subjected to 10% SDS-PAGE followed by immunoblotting with anti-Syt I (first panel), anti-Syt IV (second panel), anti-Syt IX (third panel), or HRP-conjugated anti-FLAG tag antibody (fourth panel). Input means one-eightieth the volume of the reaction mixtures used for immunoprecipitation.

RESULTS

Synaptotagmin VII-GFP in PC12 cells Is Predominantly Localized on the Dense-core Vesicles—Although several independent groups have recently proposed that Syt VII regulates Ca\(^{2+}\)-dependent vesicle exocytosis, its precise subcellular localization (plasma membrane, secretory granules, or lysosomes) is a matter of controversy (32, 36, 38, 40, 41, 43). Because most previous studies on the function of Syt VII used in vitro binding experiments (55, 58–62). To confirm the dense-core vesicle localization of Syt VII-GFP molecules, we performed an immunoelectron microscopic analysis with anti-GFP antibody. As anticipated, immunogold signals were clearly associated with almost all of the dense-core vesicles in the neurites of the PC12 cell line (Fig. 3A), although some Syt VII-GFP signals were also found in other intracellular membrane structures. We also discovered that few Syt VII-GFP signals were associated with the plasma membrane.
An N-terminal antibody-uptake experiment in the absence of high KCl stimulation (or low KCl stimulation) also supported this observation (see also Fig. 5 below). We therefore concluded that stably expressed Syt VII-GFP molecules are mainly targeted to the dense-core vesicles of PC12 cells rather than to their plasma membrane. Although the Syt VII-GFP fluorescence was partially overlapped with dextran (a marker for lysosomes; Fig. 4, P–R), consistent with a previous report (32), we found that Syt VII-GFP protein also partially overlapped with TGN38, a marker for the TGN (Fig. 4, D–E), transferrin receptor (a marker for endosomes, Fig. 4, G–H), EEA1 (a marker for early endosomes, Fig. 4, J–K), or BiP (a marker for the endoplasmic reticulum, Fig. 4, M–O). The TGN localization of Syt VII-GFP molecules was also confirmed by the immunoelectron microscopic analysis. The immunogold signals for Syt VII-GFP were associated with the membranes of the TGN (Fig. 3B, shaded arrowheads), rather than those with the cis-Golgi (black arrowheads) or the trans-Golgi (black arrows). The localization of Syt VII-GFP in the TGN is not surprising because dense-core vesicles are thought to be formed from the TGN (i.e. budding from the TGN).

**Synaptotagmin VII-GFP Is Localized on Mature Dense-core Vesicles That Undergo Ca^{2+}-dependent Exocytosis in PC12 Cells**—Because transiently overexpressed recombinant Syt VII molecules have been shown to remain in the plasma membrane...
during dense-core vesicle exocytosis and endocytosis (42, 43), we next sought to determine whether Syt VII-GFP-containing dense-core vesicles are fully mature enough to undergo regulated exocytosis in response to Ca\(^{2+}\)/H11001-stimulation. To do so, we performed an N-terminal antibody-uptake experiment as described previously (25, 45, 50). As anticipated, both anti-FLAG tag antibody (Fig. 5, red signals) and anti-Syt IX-N antibody (blue signals) were incorporated into the cell bodies and neurites of the stable PC12 cell line in a high KCl-dependent manner (Fig. 5, A–I and K–O), although faint FLAG signals (i.e. Syt VII-GFP) were detected around the edge of the cells even under low KCl conditions (Fig. 5, E–G and M–O). The uptake of the antibodies against the extracellular domain of Syt was Ca\(^{2+}\)-dependent, but not membrane depolarization-dependent, because antibody uptake did not occur in response to high KCl stimulation in the extracellular presence of 5 mM EGTA (45). Because the anti-FLAG tag antibody was unable to attach to the plasma membrane of the wild-type PC12 cells (45, 63), a small population of Syt VII-GFP molecules is present at the plasma membrane (i.e. the extracellular domain of the Syt VII-GFP molecule is exposed to the extracellular space), consistent with the results of the immunoelectron microscopic analysis described above (Fig. 3A, arrowheads). Interestingly, anti-FLAG tag antibody uptake sometimes occurred independently of anti-Syt IX-N antibody uptake (Fig. 5, I–K, arrowheads), although anti-FLAG tag and anti-Syt IX-N antibodies were almost always incorporated into the same sites.

**Fig. 5.** Ca\(^{2+}\)-dependent uptake of anti-FLAG tag antibody in undifferentiated (A–H) and \(\beta\)-nerve growth factor-differentiated PC12 cells (I–P) stably expressing Syt VII-GFP. High KCl-dependent (A–C and I–K) or low KCl-dependent (E–G and M–O) uptake of anti-FLAG tag and anti-Syt IX-N antibodies in the PC12 cell line were measured as described previously (25, 45, 50). A, E, I, and M indicate uptake of anti-FLAG tag mouse antibody (red), and B, F, J, and N indicate uptake of anti-Syt IX-N antibody (blue). D, H, L, and P correspond to the total Syt VII-GFP (GFP fluorescence) of A–C, E–G, I–K, and M–O, respectively. C, G, K, and O, are superpositions of A and B, E and F, I and J, and M and N, respectively. Note that in most cases uptake of anti-FLAG tag (red) and anti-Syt IX-N antibodies (blue) occurred at the same sites, although some red signals were clearly different from blue signals (arrowheads in I–K). Scale bars = 10 μm.

**Synaptotagmin VII-GFP Is Involved in Ca\(^{2+}\)-regulated Exocytosis of Dense-core Vesicles in PC12 Cells**—Finally, we investigated whether exogenously expressed Syt VII-GFP molecules are involved in dense-core vesicle exocytosis by means of the NPY cotransfection assay (52, 55). As shown in Fig. 6B, three independent Syt VII-GFP-expressing cell lines exhibited increased high KCl-dependent NPY secretion activity compared...
with the wild-type PC12 cells (an ~4-fold greater release of NPY), although the level of expression of NPY did not differ between the wild-type and stable PC12 cells (data not shown). Low KCl-dependent (or basal) NPY secretion from the stable cell lines was also increased compared with the wild-type PC12 cells, but the amount of basal NPY secretion from Syt VII-GFP-expressing cell lines secrete NPY more efficiently than the wild-type PC12 cells. The NPY-T7-GST secretion assay was performed as described previously (52, 55). The results are expressed as percentages of high KCl-dependent NPY secretion from the wild-type PC12 cells. L, low KCl-dependent NPY secretion (open bars); H, high KCl-dependent secretion (closed bars). C, effect of pSilencer-Syt VII on high KCl-dependent NPY secretion from the wild-type PC12 cells and Syt VII-GFP-expressing PC12 cell lines. The results are normalized by high KCl-dependent NPY secretion in control samples (closed bars). Bars indicate the means ± S.E. of three determinations. The results shown are representative of at least three independent experiments. Note that Syt VII-GFP silencing resulted in a dramatic reduction in high KCl-dependent NPY secretion from the wild-type PC12 cells (hatched bars), whereas the same siRNA slightly reduced high KCl-dependent NPY secretion from the wild-type PC12 cells (shaded bars). **, p < 0.001; *, p < 0.01, Student's unpaired t test.
Syt VII functions as a plasma membrane Ca\(^{2+}\) sensor. Syt VII transiently overexpressed in PC12 cells is mainly 60% of the siRNA-untreated control cells, which is secretion from the stable PC12 cell lines was dramatically

A level of expression of Syt VII-GFP protein was dramatically

membrane of PC12 cells (supplemental Fig. 2), whereas endog-

uous Syt VII-GFP molecules by means of a recently developed RNAi technol-

gy. To knock down all of the Syt VII splicing variants (α, β, and γ isoforms) (38, 40), a 19-nucleotide sequence in the regions common to all Syt VII splicing isoforms, i.e. the transmembrane region, was selected. When the specific siRNA targeted against Syt VII was expressed in the stable PC12 cell lines, the level of expression of Syt VII-GFP protein was dramatically reduced without affecting the levels of expression of endoge-

uous Syts I, IV, and IX proteins (Fig. 6A) or other proteins (e.g. SNAREs, Rab5, and Sec1/Munc18 proteins) that are involved in dense-core vesicle exocytosis (29) (data not shown). When NPY was coexpressed with Syt VII siRNA, high KCl-dependent NPY secretion from the stable PC12 cell lines was dramatically reduced (~60% of the siRNA-un-treated control cells, which is approximately twice as high as the high KCl-dependent NPY secretion from the wild-type PC12 cells; Fig. 6C, shaded bars). Residual NPY release activity may be mediated by residual Syt VII-GFP molecules that were not knocked down by the siRNA treatment or other Syt isoforms (e.g. Syt IX) (29). It should be noted that the Syt VII siRNA slightly reduced high KCl-depend-

ten NPY secretion form the wild-type PC12 cells (~85–90% of the control cells; this effect was not always statistically significant under our experimental conditions), which strongly supports the previous finding that the level of endogenous expression of Syt VII protein is not so high (less than 3% of that of Syt I) (13, 26). By contrast, the siRNA targeted against Syt VII had virtually no effect on low KCl-dependent NPY secretion from either the wild-type or the stable PC12 cell lines (Fig. 6C, open bars).

DISCUSSION

Because phospholipid binding to the C2 domains of Syt VII is activated by lower concentrations of Ca\(^{2+}\) (EC\(_{50}\) value = 1–2 \(\mu\)M Ca\(^{2+}\)) than is required to activate Syt I, and the recombinant Syt VII transiently overexpressed in PC12 cells is mainly localized at the plasma membrane, it has been proposed that Syt VII functions as a plasma membrane Ca\(^{2+}\) sensor for exocytosis (38, 39, 41). Recently, however, the plasma membrane localization of Syt VII and its abundance in PC12 cells have been questioned by other groups (13, 26, 40). First, the endog-

enous expression level of Syt VII has been found to be low (less than 3% of Syt I) (13, 26), and there is no direct evidence that endogenous Syt VII molecules are present at the plasma mem-

brane (i.e. the plasma membrane localization of Syt VII mole-

ules has been shown by transient overexpression of recombi-

nent Syt VII-GFP not by immunostaining of the endogenous Syt VII protein in PC12 cells). Actually, the anti-Syt VII anti-

body from Ref. 38 stained the cytoplasm of PC12 cells rather than the plasma membrane, and a 40-kDa immunoreactive band detected by this antibody in PC12 cells was unaffected by the Syt VII siRNA treatment (supplemental Fig. 1) indicating that the 40-kDa immunoreactive band is most unlikely to be an endogenous Syt VII molecule. Second, transient overexpression of Syt isoforms often causes mislocalization of the recombinant proteins in PC12 cells, in contrast to the stable expres-

sion of Syt isoforms (45). For instance, recombinant Syt IX (or I) transiently overexpressed is often targeted to the plasma membrane of PC12 cells (supplemental Fig. 2), whereas endog-

enous Syt IX (or I) is clearly localized on dense-core vesicles, but not at the plasma membrane (25, 64). Consistent with these observations, the present study on the PC12 cell line stably expressing Syt VII-GFP clearly demonstrated that the targeting of Syt VII molecules to specific membrane compart-

ments(s) and the dynamics of Syt VII molecule during dense-

core vesicle exocytosis depend on the transfection method (transient expression versus stable expression). The results of the immunocytochemical (Figs. 1 and 4) and the immunoelec-

tron microscopic analyses (Fig. 3) clearly indicated that stably expressing Syt VII molecules in PC12 cells are actually present on dense-core vesicles that undergo Ca\(^{2+}\)-dependent exocyto-

sis. In addition, because some proportions of Syt VII-GFP mole-

cules are also present in lysosomes, Syt VII is likely to be involved in the control of lysosomal exocytosis in PC12 cells as has been shown in fibroblasts (32).

Because Syt VII is a Ca\(^{2+}\)/phospholipid or SNARE-binding protein (14, 38, 39, 41, 65, 66) like neuronal Ca\(^{2+}\) sensor Syt I and Syt VII-GFP forms a hetero-oligomer with endogenous Syts I and IX (Fig. 2F), stably expressed Syt VII-GFP molecules are likely to be involved in Ca\(^{2+}\)-dependent dense-core vesicle exocytosis in the stable PC12 cell line. Actually, all three inde-

pendent cell lines showed increased high KCl-dependent NPY secretion compared with the wild-type cells (an ~4-fold in-

crase), and selective knockdown of Syt VII-GFP with RNAi dramatically reduced high KCl-dependent NPY secretion but had no effect no low KCl-dependent NPY secretion (Fig. 6). Because the Syt VII siRNA slightly but always reduced high KCl-dependent NPY secretion from the wild-type PC12 cells (~85–90% of the control cells; 1 case of 4 was statistically significant), we speculated that endogenous Syt VII molecules are also involved in the control of Ca\(^{2+}\)-regulated dense-core vesicle exocytosis in the wild-type PC12 cells.

In summary, we have demonstrated that the Syt VII mole-

cules in the stable PC12 cell line are actually present on dense-

core vesicles and that Syt VII-containing vesicles move dynam-

ically during the exocytosis and endocytosis cycle in response to Ca\(^{2+}\) stimulation. Because stable expression of Syt VII-GFP dramatically enhances stimulated NPY secretion and two C2 domains of Syt VII bind Ca\(^{2+}\) in vitro, we proposed that Syt VII is involved in the control of dense-core vesicle exocytosis, possi-

bly functioning as a Ca\(^{2+}\) sensor on dense-core vesicles. In addition, it is also possible that Syt VII acts as a plasma membrane Ca\(^{2+}\)-sensor because some Syt VII-GFP molecules are actually present at the plasma membrane of the PC12 cell line. Our findings also strongly indicate that the results of transient overexpression of recombinant Syt proteins should be carefully evaluated to avoid misunderstanding the function and localization of Syt isoforms.

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