Nerve Growth Factor-dependent Sorting of Synaptotagmin IV Protein to Mature Dense-core Vesicles That Undergo Calcium-dependent Exocytosis in PC12 Cells*

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Synaptotagmin IV (Syt IV) is a fourth member of the Syt family and has been shown to regulate some forms of memory and learning by analysis of Syt IV null mutant mice (Ferguson, G. D., Anagnostaras, S. G., Silva, A. J., and Herschman, H. R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5598–5603). However, the involvement of Syt IV protein in vesicular trafficking and even its localization in secretory vesicles are still matters of controversy. Here we present several lines of evidence showing that the Syt IV protein in PC12 cells is normally localized in the Golgi or immature vesicles at the cell periphery and is sorted to fusion-competent mature dense-core vesicles in response to short nerve growth factor (NGF) stimulation. (i) In undifferentiated PC12 cells, Syt IV protein is mainly localized in the Golgi and small amounts are also present at the cell periphery, but according to the results of an immunocytochemical analysis, they do not colocalize with conventional secretory vesicle markers (Syt I, Syt IX, Rab3A, Rab27A, vesicle-associated membrane protein 2, and synaptophysin) at all. By contrast, limited colocalization of Syt IV protein with dense-core vesicle markers is found in the distal parts of the neurites of NGF-differentiated PC12 cells. (ii) Immunoelectron microscopy with highly specific anti-Syt IV antibody revealed that the Syt IV protein in undifferentiated PC12 cells is mainly present on the Golgi membranes and immature secretory vesicles, whereas after NGF stimulation Syt IV protein is also present on the mature dense-core vesicles. (iii) An N-terminal antibody-uptake experiment indicated that Syt IV-containing vesicles in the neurites of NGF-differentiated PC12 cells undergo Ca2+-dependent exocytosis, whereas no uptake of the anti-Syt IV-N antibody was observed in undifferentiated PC12 cells. Our results suggest that Syt IV is a stimulus (e.g. NGF)-dependent regulator for exocytosis of dense-core vesicles.

Synaptotagmin (Syt) is a family of C-terminal-type (C-type) tandem C2 proteins with an N-terminal single transmembrane domain and is thought to regulate membrane traffic (reviewed in Refs. 1–5). To date, 13 distinct syt genes have been identified in mice, rats, and humans, and several syt genes have been identified in invertebrates (6–9). Syt I is evolutionarily conserved and is the best characterized isoform of Syt. Abundant Syt I is found on synaptic vesicles, and it has been shown to be essential for synaptic vesicle exocytosis and endocytosis in neurons by genetic analysis of syt mutants (reviewed in Refs. 2–5) and by antibody or peptide inhibition experiments (10–13). Syt I is also present on dense-core vesicles in some neuroendocrine cells and has been shown to regulate Ca2+-dependent dense-core vesicle exocytosis (14–22). However, the precise subcellular localizations and functions of other Syt isoforms (Syts III–XIII) are still a matter of controversy (see Discussions in Refs. 23 and 24).

Syt IV was first described as a fourth member of the Syt family (25), and the syt IV gene was subsequently identified as an immediate early gene induced by membrane depolarization in brain and in PC12 cells (26–28). Syt IV mRNA expression is developmentally regulated (29, 30) and rapidly changes in response to a variety of extracellular stimuli (31–35). Since Syt IV null mutant mice exhibit abnormalities in motor performance and some forms of memory related to the hippocampus (36), it has been suggested that Syt IV protein is crucial to learning and memory (or synaptic plasticity) (28, 37). However, the role of Syt IV on the molecular level during learning and memory and its involvement in vesicular trafficking remain to be elucidated. Although Syt IV protein was first proposed to be a synaptic vesicle protein and function in concert with Syt I (38–40), recent subcellular fractionation studies and immunoelectron microscopy have clearly demonstrated that rather than being a synaptic vesicle protein, Syt IV is present on uncharacterized vesicular/organellar structures in both axons and dendrites and in the Golgi of developing mouse brain (27, 37, 41). Perinuclear localization of Syt IV protein and distinct subcellular localizations of Syts I and IV have also been observed in some endocrine cells (PC12, pituitary AtT-20, and pancreatic α-cells) (27, 42, 43). In addition, Syt IV protein has been shown to be localized on immature vesicles rather than mature secretory vesicles in AtT-20 cells, suggesting a role of Syt IV protein as a keeper of the switch for the change from unregulated to regulated secretory vesicles (42). Whether endogenous Syt IV-containing vesicles/organelles fuse plasma membrane in response to Ca2+; however, had never been determined despite this information being important to learning whether Syt IV acts as a positive regulator or negative regulator of exocytosis.

In this study, we observed a nerve growth factor (NGF)-de-
pendent redistribution of Syt IV protein in PC12 cells and dis
covered that NGF stimulates the sorting of Syt IV protein to
mature dense-core vesicles that undergo Ca2+-dependent exo
cytosis in NGF-differentiated PC12 cells. Based on our find-
ings, we discuss the distinct roles of Syt I and Syt IV in secre
tory vesicle trafficking in the brain.

MATERIALS AND METHODS

Antibody Purification—The anti-Syt I mouse monoclonal antibody (SYA148) was from StressGen (Victoria, British Columbia, Canada). The anti-Syt IX-C2A, anti-Syt IX-N, and anti-Syt IV-C2A rabbit polyclonal antibodies were prepared as described previously (21, 27). The anti-Syt IV-N-specific for the N-terminal domain of the mouse Syt IV (anti-
Syt IV-N) was raised against the following synthetic peptide with a
C-terminal artificial Cys residue: MAPITTSRVEFDEC (Syt IV-N amino
acids 1–14) (44). The antibody was affinity-purified by exposure to the
antigenic peptide bound to FMP-activated Cellulofine (Seikagaku Co.)
as described previously (44). The specificity of the antibody was checked
by immunoblotting with recombinant TT-tagged Syts I–XIII expressed
in COS-7 cells (21, 22, 27, 45). Under our experimental conditions,
immunoblotting did not reveal any evidence of cross-reactivity be-
tween the anti-Syt IV-N antibody and other Syt isoforms including with
closely related isoform Syt XI (46) (data not shown). The protein con-
centration was determined with a Bio-Rad protein assay kit with bovine
serum albumin as a reference. Immunoblotting was performed as de-
scribed previously (7).

The purified anti-Syt IV-N and anti-Syt IX-N antibodies were conju-
gated with carboxytetramethylrhodamine and carboxyfluorescein (Mo-
olecular Probes, Inc., Eugene, OR), respectively, according to the manu-
facturer’s instructions (11, 47).

Antibody-uptake Experiments—NGF-differentiated PC12 cells were
cultured at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium
containing 10% horse serum and 10% fetal bovine serum on 35-mm
glass-bottom dishes (MatTek Corp., Ashland, MA) coated with collagen
type IV (BD Biosciences Labware) (27, 45). After washing twice with
phosphate-buffered saline, the cells were stimulated for 10 min at 37 °C
with low KCl buffer (5.6 mM KCl, 145 mM NaCl, 2.2 mM CaCl2, 0.5 mM
MgCl2, 5.6 mM glucose, and 15 mM HEPES-KOH, pH 7.4) or high KCl
buffer (56 mM KCl, 95 mM NaCl, 2.2 mM CaCl2, 0.5 mM MgCl2, 5.6 mM
glucose, and 15 mM HEPES-KOH, pH 7.4) containing either the rho-
damine-labeled anti-Syt IV-N or fluorescein-labeled anti-Syt IX-N an-
tibodies (10 μg/ml) (6, 21, 25). The cells were immediately washed twice
with phosphate-buffered saline and then fixed in 4% paraformaldehyde
in 0.1 mM sodium phosphate buffer for 20 min at room temperature as
described previously (27, 45). The incorporated antibodies were directly
analyzed with a fluorescence microscope (TE300, Nikon, Tokyo, Japan)
attached to a laser confocal scanner unit CSU 10 (Yokogawa Electric
Corp., Tokyo, Japan) and HISC A CCD camera (C5780, Hamamatsu
Photonics, Hamamatsu, Japan) or a confocal fluorescence mi-
croscope (FluoView, Olympus, Tokyo, Japan). Images were pseudo-
colored and superimposed with Adobe Photoshop software (version 7.0).

Immunoelectron Microscopy—The pre-embedding silver enhance-
ment immunogold method was performed as described by Yoshimori
et al. (48) with a slight modification. PC12 cells cultured on collagen
type IV-coated plastic coverslips were fixed in 4% paraformaldehyde in
0.1 M sodium phosphate buffer for 20 min at room temperature as
described previously (21, 46). The cells were then exposed to the anti-
Syt IV-C2A rabbit IgG (1/500 dilution) in the blocking solution overnight.
After washing in PB containing 0.005% saponin six times for 10 min, cells
were incubated with the Fab’ fragment of goat anti-rabbit IgG that had
been conjugated to colloidal gold (1.4-nm diameter) in the blocking solution
for 2 h. The cells were then washed with PB six times for 10 min and fixed with 1% glutaraldehyde in PB for 10 min. After washing, the cells
were dehydrated with a graded series of ethanol and embedded in
toepoxy resin. Ultrathin sections were doubly stained with 2% uranyl
acetate and lead citrate.

Miscellaneous Procedures—Immunocytochemical analysis of NGF-
differentiated PC12 cells was also performed as described previously
(27, 45). Afterward, antibodies were used for immunocytochemical anal-
ysis: anti-Syt I (SYA148, 1:250 dilution, StressGen), anti-Syt IX (1:100 dilution, Transduction Laboratories, Lexington, KY), anti-Rab3A (1:500 dilution, Transduction Laboratories), anti-Rab27A (1:1000 dilution, Transduction Laboratories), anti-VAMP-2 (1:1000 dilution, Synaptic Systems, Göttingen, Germany), anti-synaptophysin mouse monoclonal antibodies (1:250 dilution, Sigma), and anti-Syt IV-C2A rabbit polyclonal antibody (1.5 μg/ml) (27). CGA4–8 cells and their cell lysates were prepared as described previously (49). SDS-PAGE and immuno-
blotting analyses were also performed as described previously (7). Im-
munoreactive bands were captured by Gel Print 2000i/VGA and ana-
lyzed with Basic Quantifier software (version 1.0) (BioImage) as described previously (50).

RESULTS

Less Colocalization of Synaptotagmin IV Protein with Conven-
tional Secretory Vesicle Markers in NGF-differentiated
PC12 Cells—In a previous study using Syt IV-specific antibody,
we showed that endogenous Syt IV protein is mainly present in
brefeldin A-sensitive perinuclear regions (probably the Golgi) of
undifferentiated PC12 cells (27, 51). However, when rela-
tively high concentrations of the anti-Syt IV-C2A antibodies
(>1.5 μg/ml) were used for immunocytochemical analysis, we
also detected weak dot-like Syt IV signals at the cell periphery
(Fig. 1 in green), and in some cells, the Syt IV signals were
accumulated at the tips of the cellular processes (Fig. 1A, arrowhead).
To investigate whether Syt IV signals at the cell
periphery correspond to conventional secretory vesicles (dense-
core vesicles and/or synaptic-like microvesicles), we compared
the Syt IV signals with six different secretory vesicle markers
(21, 24, 27, 52) (Fig. 1 in red; Rab27A (B and C), Rab3A (E and
F), Syt I (H and I), Syt IX (K and L), synaptophysin (N and O),
and VAMP-2 (data not shown)). To our surprise, none of the
secretory vesicle markers tested colocalized with Syt IV protein
even in the cellular processes (Fig. 1C, inset), indicating that
Syt IV protein is unlikely to be present on conventional secre-
tory vesicles in undifferentiated PC12 cells (Fig. 1C, F, I, L, and
O) (53).

Consistent with this finding, we found that the Syt IV expression
levels in the CGA4–8 cell lines that almost completely
lack dense-core vesicles (<10% of the number in wild-type
PC12 cells) as a result of antisense knock down of chromogranin
A (49) were almost unchanged (>80% control cell level). By
contrast, the expression of Syts I and IX, two major Syt iso-
forms abundantly expressed on dense-core vesicles (21, 22),
was dramatically reduced to ~20–30% of their expression lev-
els in the control cells (data not shown).

After exposing the PC12 cells to NGF, we observed that Syt IV
protein was also localized in the distal parts of neurites
where dense-core vesicles are known to be accumulated (Fig. 2
in green), although the majority of the Syt IV signals remained
in the perinuclear region. We again compared Syt IV signals
with the six secretory vesicle markers, especially focusing on
the distal portions of neurites (Fig. 2, insets). It should be noted
that only a small population of Syt IV signals colocalized with
dense-core vesicle markers (yellow dots in Fig. 2, panels C, F, I,
and L) and none with synaptic-like microvesicle markers (Fig. 2O,
synaptophysin, inset), although the majority of the Syt IV
signals (green) in the neurites still did not coincide well with
the dense-core vesicle markers (red) (Fig. 2, panels C, F, I, and
L, insets). These observations were in great contrast to Syt I
and Syt IX, two major Syt isoforms in PC12 cells that colocalize
well in the neurites (21). Because NGF did not increase the
protein expression levels of Syt IV (27), the Syt IV protein in the
distal parts of neurites may have been transported from the
cell body (i.e. newly forming vesicles from the TGN (trans-Golgi
network)) or may have been redistributed locally (i.e. sorting of
immature vesicles to dense-core vesicles at the cell periphery)
but were unlikely to have been synthesized locally de novo.
Therefore, we hypothesized that some populations of Syt IV
protein are sorted to dense-core vesicles in response to NGF stimulation, and we tested this hypothesis by immunoelectron microscopy (Fig. 3) because it is impossible to judge the localization of Syt IV protein on dense-core vesicles by immunocytochemistry alone.

**Synaptotagmin IV Protein Is Sorted to Dense-core Vesicles in PC12 Cells after NGF Stimulation**—Immunoelectron microscopic analysis was then performed with highly specific anti-Syt IV antibody to reveal the exact localization of Syt IV protein in PC12 cells (27, 37). As shown in Fig. 3A, Syt IV protein was abundantly localized on the Golgi membrane in undifferentiated PC12 cells, the same as in neocortical neurons of the developing mouse brain (37). The Syt IV signals were prominent in the cisterns of the trans-Golgi, TGN, and the vacuoles presumably formed from TGN, but some signals were also observed in the cis-Golgi. Consistent with the immunocytochemical findings described above, there were virtually no Syt IV signals on the mature dense-core vesicles around the plasma membrane (Fig. 3A, upper inset, arrowheads), but in some cases, the Syt IV signals were observed on the lighter vesicles presumably corresponding to immature vesicles (Fig. 3A, lower inset, arrowheads) (42). The majority of the Syt IV signals in NGF-differentiated PC12 cells was still observed around the Golgi membranes (Fig. 3B), but some Syt IV signals were clearly localized on the dense-core vesicles in addition to the immature vesicles, especially in the distal parts of neurites and around the plasma membrane (Fig. 3B, arrowheads, inset).

Interestingly, in many specimens, the Syt IV-containing dense-core vesicles were much lighter than the non-Syt IV-containing dense-core vesicles, suggesting that Syt IV-containing dense-
core vesicles may form immediately after NGF stimulation. Therefore, we concluded that at least some populations of Syt IV protein are indeed sorted to dense-core vesicles in PC12 cells after NGF stimulation.

**NGF-dependent Sorting of Synaptotagmin IV Protein to Mature Dense-core Vesicles That Undergo Ca^{2+}-dependent Exocytosis**—Finally, we attempted to determine whether Syt IV-containing dense-core vesicles in the presence of NGF are fully mature and thus capable of exocytosis in response to Ca^{2+} stimulation because PC12 cells contain various types of secretory vesicles (54). To visualize the dynamics of endogenous Syt IV molecules during Ca^{2+}-dependent exocytosis, an N-terminal antibody-uptake experiment was performed as described previously (21, 23). Antibodies against the luminal domain of Syt IV (or IX) were added to the extracellular medium, and PC12 cells were stimulated either with a low or high concentration of KCl. If the Syt IV-containing vesicle undergoes exocytosis in response to Ca^{2+} stimulation, the N terminus of Syt IV would be accessible on the outer surface of the cell membrane and therefore should be recognized by anti-Syt IV-N antibody in the culture medium. The Syt IV-antibody complex would then be incorporated into the cell by endocytosis.

The uptake of the anti-Syt IX-N antibody into the cell body and neurites occurred only at depolarizing KCl concentrations regardless of NGF exposure as described previously (Fig. 4, I–L). The high KCl-dependent uptake of the antibody should be Ca^{2+}-dependent but not depolarization-dependent, because no uptake was observed in the presence of extracellular EGTA even in response to high KCl stimulation (data not shown) (23). By contrast, the uptake of the anti-Syt IV-N antibody was both NGF- and high KCl-dependent (Fig. 4, A–H). The anti-Syt IV-N antibody was not incorporated very much into the cell body of undifferentiated PC12 cells even in response to the high KCl stimulation (Fig. 4, A–D), consistent with our immunoelectron microscopic observations that Syt IV protein is mainly localized in the Golgi or immature secretory vesicles in undifferentiated PC12 cells (Fig. 3A). It should be noted that after NGF stimulation, high KCl-dependent uptake of the anti-Syt IV-N antibody into neurites was prominent (Fig. 4, E–H).

We also used fluorescence-labeled antibodies (i.e. rhodamine-labeled anti-Syt IV-N and fluorescein-labeled anti-Syt IX-N antibodies) to investigate whether Syt IV and Syt IX proteins are incorporated into the same sites or different sites via endocytosis. There was obvious colocalization of the fluorescein-anti-Syt IX-N and rhodamine-anti-Syt IV-N antibodies in the neurites, but some signals were Syt IX-N antibody-specific (Fig. 5, A–C, arrows) or Syt IV-N antibody-specific (Fig. 5, A–C, arrowheads). It should be noted that colocalization of Syt IX
and Syt IV clearly increased after high KCl stimulation (compare Fig. 2 with 5). This change may be explained by the notion that even if Syt IV- and Syt IX-containing vesicles undergo exocytosis at different sites, Syt IV and Syt IX proteins can be retrieved at the same sites and sorted to the same dense-core vesicles.

We further investigated whether the Syt IV-N antibody uptake occurs after only a short exposure to NGF, and the results showed that only a 1-h exposure to NGF is adequate to detect the uptake of the rhodamine-Syt IV-N antibody (Fig. 5F), suggesting that Syt IV proteins at the cell periphery may be rapidly sorted into dense-core vesicles rather than being formed from the TGN and transported to the cell periphery. Unlike the N-terminal antibody uptake seen in the neurites of NGF-differentiated PC12 cells, the fluorescein-Syt IX-N and rhodamine-Syt IV-N antibody signals were often somewhat different (Fig. 5G, insets).

**DISCUSSION**

Although both the Syt IV mRNA and protein expression levels rapidly increase after exposure to depolarizing stimuli (26, 27) and Syt IV null mutant mice exhibit abnormalities in some forms of memory and motor performance (36), whether Syt IV is actually involved in membrane traffic related to learning and memory had never been elucidated. All of the previous studies on the function of Syt IV protein had been conducted by overexpression (39, 53) or exogenous addition of recombinant proteins (40). The former approach is sometimes unreliable for studies on Syt function because exogenously expressed Syt proteins or fragments (especially produced by forced overexpression) often result in mislocalization when compared with endogenous protein (23, 24, 55). Actually, one study (53) reports that overexpressed Syt IV protein is localized on dense-core vesicles in undifferentiated PC12 cells, whereas others show (42, 43, 51) that it is localized in Golgi and/or immature vesicles. The latter approach (so-called “dominant negative approach”) also has some drawbacks, because recombinant proteins from Syt isoforms that are not endogenously expressed inhibit Ca\(^{2+}\)-dependent secretion in PC12 cells more strongly than recombinant proteins from endogenous Syt isoforms (22, 56) and recombinant Syt proteins bind various mol-

**FIG. 4.** NGF-dependent sorting of synaptotagmin IV protein to mature dense-core vesicles that undergo Ca\(^{2+}\)-dependent exocytosis in PC12 cells. Panels A, E, I, high KCl (56 mM) dependent uptake of anti-Syt IV-N (A and E) and anti-Syt IX-N (I) antibodies. Panels C, G, and K, low KCl (5.6 mM) dependent uptake of anti-Syt IV-N (C and G) and anti-Syt IX-N (K) antibodies (21, 23). Panels B, D, F, H, J, and L, light-field image of A, C, E, G, I, and K, respectively. Panels A–D and I–L, undifferentiated PC12 cells. Panels E–H, neurites of NGF-differentiated PC12 cells. Scale bar indicates 20 μm.

**FIG. 5.** Uptake of rhodamine-labeled anti-Syt IV-N and fluorescein-labeled anti-Syt IX-N antibodies in NGF-differentiated PC12 cells. Panels A–D, high KCl-dependent uptake of rhodamine-anti-Syt IV-N (red in B) and fluorescein-anti-Syt IX-N antibodies (green in A) in the neurites of NGF-differentiated PC12 cells. C, a superposition of A and B. D, light-field image of A–C. Note that most of the rhodamine-anti-Syt IV-N and fluorescein-anti-Syt IX-N antibodies were incorporated into the same sites, although some signals were rhodamine-anti-Syt IV-N-specific (arrowheads) or fluorescein-anti-Syt IX-N antibody-specific (arrows). Panels E–G, high KCl-dependent uptake of rhodamine-anti-Syt IV-N (red in F) and fluorescein-anti-Syt IX-N antibodies (green in E) in PC12 cells after a 1-h exposure to NGF. Note that some of the rhodamine-anti-Syt IV-N and fluorescein-anti-Syt IX-N antibodies were incorporated into somewhat different sites (insets in G). Scale bar indicates 20 μm.

ecules important for Ca\(^{2+}\)-dependent secretion (e.g. SNARE protein) (18, 19). In addition, recombinant C2 fragments from bacteria are often contaminated by non-proteinaceous components (57), and contradictory results have been reported even when the same cDNA constructs have been used (18, 56). Therefore, it is crucial to determine the exact localization of endogenous Syt IV protein and its dynamics during Ca\(^{2+}\)-dependent exocytosis. In this study, we used PC12 cells to study Syt IV function and localization, because Syt IV is more abundantly expressed in PC12 cells than in brain (27) and PC12 cells are often used as a good system for studying Ca\(^{2+}\)-dependent exocytosis.
Immunoelectron microscopic analysis (Fig. 3) and the N-terminal antibody-uptake experiment (Fig. 4) clearly demonstrated that the endogenous Syt IV protein in PC12 cells is mainly localized in the Golgi and/or immature secretory vesicles (42) and that after NGF stimulation some populations of Syt IV protein are sorted to mature dense-core vesicles. Because the Syt IV N-terminal antibody uptake occurs only after a 1-h exposure to NGF, Syt IV protein present at the cell periphery rather than TGN-derived Syt IV protein is most likely to be sorted to mature dense-core vesicles. Since exposure to NGF did not alter the expression levels of Syts I and IV (27), newly formed Syt IV-containing dense-core vesicles are expected to carry a single Syt IV isoform, not Syts I and IX. Consistent with this finding, colocalization of Syts I (or IX) and IV in the distal parts of neurites was very limited even in NGF-differentiated PC12 cells (Fig. 2, I and L). However, after exocytosis, Syt IV and Syt IX proteins are likely to be retrieved at the same or similar sites and then be sorted to the same dense-core vesicles because of the obvious colocalization of Syt IV and IX after high KCl stimulation (i.e., colocalization of the fluorescent anti-Syt-IX-N and rhodamine-anti-Syt-IV-N antibodies in the neurites) (Fig. 5, A–C). Consistent with our findings, Ng et al. (58) and Amino et al. (59) recently reported that short NGF stimulation of PC12 cells enhances releasable pools of peptide hormone. Thus, it is highly possible that Syt IV is involved in the enhancement of some releasable pools in response to NGF.

At present, the mechanism of the NGF-dependent Syt IV sorting to mature dense-core vesicles at the cell periphery remains unknown, but we speculate that certain properties (e.g., phosphorylation and/or protein interaction) in the unique spacer domain of Syt IV (51) qualitatively change after NGF exposure. We recently suggested (60) that the interaction between Syt I and VAMP-2 may be involved in sorting of Syt I protein to secretory vesicles. Thus, it may be possible that interaction between Syt IV and other VAMP isoforms (e.g., VAMP-4 that is also localized in the Golgi and immature secretory vesicles) (42) regulates Syt IV protein sorting since Syt IV interacts with certain VAMP isoforms in vitro.2 The physiological meaning of the Syt-VAMP interaction is now under investigation in our laboratory.

How does Syt IV protein function during Ca2+-dependent exocytosis? Based on an analysis of Syt IV overexpression in Drosophila exocytosis? Based on an analysis of Syt IV overexpression in Drosophila and in vivo, the physiological meaning of the Syt-VAMP interaction is now under investigation in our laboratory.

In summary, we showed for the first time that Syt IV protein is NGF-dependently sorted to fusion-competent mature dense-core vesicles in PC12 cells. Our results suggest that Syt IV protein regulates stimulus (e.g., NGF)-dependent membrane trafficking that may be involved in plastic changes at the synapses in brain in contrast to the role of Syt I protein in synaptic vesicle trafficking.

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