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N-Glycosylation Is Essential for Vesicular Targeting of Synaptotagmin 1

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Summary

Synaptotagmins 1 and 7 are candidate Ca^{2+} sensors for exocytosis localized to synaptic vesicles and plasma membranes, respectively. We now show that the N-terminal intraluminal sequence of synaptotagmin 1, when transplanted onto synaptotagmin 7, redirects synaptotagmin 7 from the plasma membrane to secretory vesicles. Conversely, mutation of the N-terminal N-glycosylation site of synaptotagmin 1 redirects synaptotagmin 1 from vesicles to the plasma membrane. In cultured hippocampal neurons, the plasma membrane-localized mutant of synaptotagmin 1 suppressed the readily releasable pool of synaptic vesicles, whereas wild-type synaptotagmin 1 did not. In addition to the intraluminal N-glycosylation site, the cytoplasmic C_2 domains of synaptotagmin 1 were required for correct targeting but could be functionally replaced by the C_2 domains of synaptotagmin 7. Our data suggest that the intravesicular N-glycosylation site of synaptotagmin 1 collaborates with its cytoplasmic C_2 domains in directing synaptotagmin 1 to synaptic vesicles via a novel N-glycosylation-dependent mechanism.

Introduction

Synaptic vesicles are specialized organelles whose only known function is to mediate neurotransmitter release by Ca^{2+}-triggered exocytosis. In order to undergo exocytosis, synaptic vesicles must assemble in presynaptic nerve terminals, fill with neurotransmitters, and dock at the active zone (reviewed in Südhof, 1995). Furthermore, for sustained release during repetitive stimulation, synaptic vesicles must undergo rapid endocytosis and recycling after exocytosis. Thus, the biogenesis, maintenance, and recycling of synaptic vesicles are as important for overall synaptic transmission as exocytosis. Much progress has been made in the understanding of neurotransmitter uptake and synaptic vesicle exo- and endocytosis (reviewed in Slepnev and De Camilli, 2000; Fon and Edwards, 2001; Harris et al., 2001; Richmond and Broadie, 2002; Jahn et al., 2003). Little is known, however, about the biogenesis of synaptic vesicles, the sorting of their protein components, and the maintenance of synaptic vesicles as separate organelles during vesicle recycling.

Synaptotagmins constitute a family of neuronal proteins that are characterized by a common domain structure (reviewed in Adolfsen and Littleton, 2001; Südhof, 2002; Tokuoka and Goda, 2003). All synaptotagmins contain an N-terminal transmembrane region that is preceded by a short noncytoplasmic (i.e., extracellular and/or intraluminal) sequence, a central linker, and two C-terminal C_2 domains. The C_2 domains account for the majority of the synaptotagmin sequences and are their only conserved domains. Most synaptotagmins are Ca^{2+} binding proteins in which the C_2 domains act as effector domains (reviewed in Rizo and Südhof, 1998), although probably not all synaptotagmins bind Ca^{2+}. Synaptotagmin 1, the best characterized and most abundant synaptotagmin, is a Ca^{2+} sensor for the fast component of Ca^{2+}-triggered exocytosis in vertebrate neurons (Geppert et al., 1994), neuroendocrine cells (Voets et al., 2001), and *Drosophila* neurons (Yoshihara and Littleton, 2002). This function involves binding of Ca^{2+} to the C_2 domains of synaptotagmins because changing the apparent Ca^{2+} affinity of synaptotagmin 1 causes an equivalent change in the Ca^{2+} affinity of release (Fernández-Chacón et al., 2001).

Besides synaptotagmins 1 and 2 (which is closely related to synaptotagmin 1 and probably functions as a fast Ca^{2+} sensor in synapses in caudal brain regions that lack synaptotagmin 1 [Geppert et al., 1991]), other synaptotagmins—in particular synaptotagmins 3, 5, 7, and 9—may act as Ca^{2+} sensors for exocytosis, possibly for the slow component of release (Li et al., 1995a, 1995b; Sugita et al., 2001; Fukuda et al., 2002; Saegusa et al., 2002; Shin et al., 2002). In brain, synaptotagmins 1 and 2 were only detected on synaptic vesicles and secretory granules (Matthew et al., 1981; Perin et al., 1991; Walch-Solimena et al., 1993), but synaptotagmins 3 and 7 were localized to the synaptic plasma membrane (Butz et al., 1999; Sugita et al., 2001). The presence of complementary Ca^{2+} sensors with different Ca^{2+} binding properties on the vesicular versus plasma membranes during exocytosis raised the possibility that synaptotagmins 1 and 7, together with other synaptotagmins, control exocytosis by distinct Ca^{2+}-dependent reactions centered on opposing membranes. However, the notion that synaptotagmins 1 and 7 may function as complementary Ca^{2+} sensors has been questioned. In studies of fibroblast growth factor 1 secretion, synaptotagmin 1 was proposed to be an essential ubiquitous plasma membrane protein that mediates nonclassical secretion (LaVallee et al., 1998; Prudovsky et al., 2002). In studies of lysosome exocytosis, synaptotagmin 7 was characterized as a ubiquitous lysosomal protein (Martinez et al., 2000; Reddy et al., 2001). In neurons and neuroendocrine cells, the detailed examination of synaptotagmins 1 and 7 using subcellular fractionation, transfections of EYFP-tagged proteins, immunofluorescence, and im-

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munogold electron microscopy seems to firmly establish their localizations (Matthew et al., 1981; Perin et al., 1990, 1991; Walch-Solimena et al., 1993; Sugita et al., 2001, 2002). In peripheral tissues, however, the expression and localization of synaptotagmins 1 and 7 have not been studied in detail. It is thus possible that these synaptotagmins assume distinct localizations in neuronal and nonneuronal cells, especially since, in nonneuronal cells, transfected synaptotagmin 1 is present on the plasma membrane (Feany et al., 1993).

A further critical question about synaptotagmins 1 and 7 regards the mechanism that mediates their differential localizations to synaptic vesicles versus plasma membranes. No obvious sequence differences between synaptotagmins 1 and 2 versus synaptotagmins 3 and 7 exist that could explain these localizations. The differential targeting of synaptotagmins was reproduced in transfected neuroendocrine PC12 cells (Sugita et al., 2001, 2002) where it is caused by the selective endocytosis of synaptotagmin 1 but not synaptotagmin 7 from the plasma membrane (Dasgupta and Kelly, 2003). Based on these results, it was proposed that internalization signals and inhibitory sequences that are embedded in the C_2 domains determine the localizations of synaptotagmins 1 and 7 (Blagoveshchenskaya et al., 1999; Jarousse and Kelly, 2001; Dasgupta and Kelly, 2003). However, the C_2 domains of synaptotagmins 1 and 7 are very similar, and both bind to AP2, which may couple synaptotagmins to endocytosis (Zhang et al., 1994; Li et al., 1995a). A potentially confounding factor in previous studies on the localization signals of synaptotagmins (Blagoveshchenskaya et al., 1999; Jarousse and Kelly, 2001; Dasgupta and Kelly, 2003) is that all experiments were performed with fusion proteins in which the isoform-specific N-terminal sequences of synaptotagmins were removed. It is thus unclear whether the similar C_2 domains of synaptotagmins 1 and 7 are sufficiently different to specify their distinct localizations.

In the present experiments, we have examined the molecular determinants that mediate the internalization and targeting of synaptotagmin 1. Our data identify the unique intraluminal N-glycosylation site of synaptotagmin 1 as an essential determinant of its vesicular localization, whereas the C_2 domains of synaptotagmins 1 and 7 are functionally exchangeable. Furthermore, we demonstrate the functional importance of synaptotagmin 1 N-glycosylation for synaptic transmission. Our observations are unexpected in view of the fact that most sorting signals are thought to be cytoplasmic and provide a rationale for the exclusive N-glycosylation of vesicular synaptotagmins 1 and 2 in vertebrates.

Results

Developmental Tissue-Specific Expression of Synaptotagmins

The reported brain-specific expression of synaptotagmin 1 (Perin et al., 1990) conflicts with a suggested ubiquitous function in protein secretion (Prudovsky et al., 2002). Furthermore, the presence of synaptotagmin 7 mRNA in peripheral tissues (Li et al., 1995a) and of synaptotagmin 7 protein in nonneuronal cells (Martinez et al., 2000) is apparently at odds with the enrichment of synaptotagmin 7 in adult brain (Sugita et al., 2001). To address these issues, we raised multiple antibodies to synaptotagmins 1 and 7 and examined their tissue-specific expression during development (Figure 1A). We found that, at all developmental stages, synaptotagmin 1 is detectable in mice only in brain but not in heart, kidney, lung, or liver. In contrast, synaptotagmin 7 was abundant in all embryonic tissues tested. Postnatally, however, synaptotagmin 7 expression outside of brain decreased to low levels but increased in brain (Figure 1A). The prenatal ubiquitous form of synaptotagmin 7 consisted of single, small splice variant, whereas postnatally expressed brain synaptotagmin 7 became diversified into a large number of splice variants (Figure 1A; Sugita et al., 2001). Thus, the expression pattern of synaptotagmin 7 protein is strictly dependent on the developmental stage of the tissue, but in contrast to that of synaptotagmin 1, synaptotagmin 7 is not restricted to brain.

The expression of synaptotagmin 7 in developing, growing tissues and in postmitotic neurons suggests that, although synaptotagmin 7 is not neuron specific, it is also not a universal cellular component. Do rapidly growing nonneuronal cell lines express synaptotagmin 7 similar to immature tissues? To address this question, we examined a series of tissue culture cell lines by immunoblotting (Figure 1B). Most cell lines (including PC12 cells that were reported to lack synaptotagmin 7; see Zhang et al., 2002) expressed considerable amounts of the short splice variant of synaptotagmin 7 (Figure 1B), whereas synaptotagmin 1 was detected only in PC12 cells. In these experiments, we employed four independent synaptotagmin 7 antibodies to ensure that the detected synaptotagmin 7 protein of 45 kDa truly corresponds to synaptotagmin 7 (Figure 1B). This was necessary because the 45 kDa protein we detected differs from the 70 kDa band that was identified in nonneuronal cells with an antibody that localized synaptotagmin 7 to lysosomes (Martinez et al., 2000). All of our antibodies to synaptotagmin 7 detected only the 45 kDa band in nonneuronal cells (Figures 1A and 1B), except for the antibody to the neuron-specific long splice variant that detects no protein in nonneuronal cells. These data suggest that the lysosomal 70 kDa protein of Martinez et al. (2000) and the plasma membrane synaptotagmin 7 protein detected with our antibodies (Sugita et al., 2001, and Figure 1B) are different.

Differential Targeting of Synaptotagmins in Neuroendocrine versus Nonneuronal Cells

To establish an assay for the targeting sequences for synaptotagmins, we expressed synaptotagmins 1 and 7 in neuroendocrine and nonneuronal cells (PC12 and AtT-20 versus HEK293 and BHK21 cells). The two synaptotagmins were produced as C-terminally tagged EYFP fusion proteins to allow direct visualization of the respective proteins. We found that, in nonneuronal cells, synaptotagmins 1 and 7 were both localized to the plasma membrane (Figure 2A). In neuroendocrine cells, by contrast, transfected synaptotagmin 1 was present in intracellular vesicles, whereas synaptotagmin 7 continued to be on the plasma membrane. The intracellular vesicles containing transfected synaptotagmin 1 corresponded to secretory granules since synaptotagmin 1-EYFP precisely colocalized with cotransfected atrial

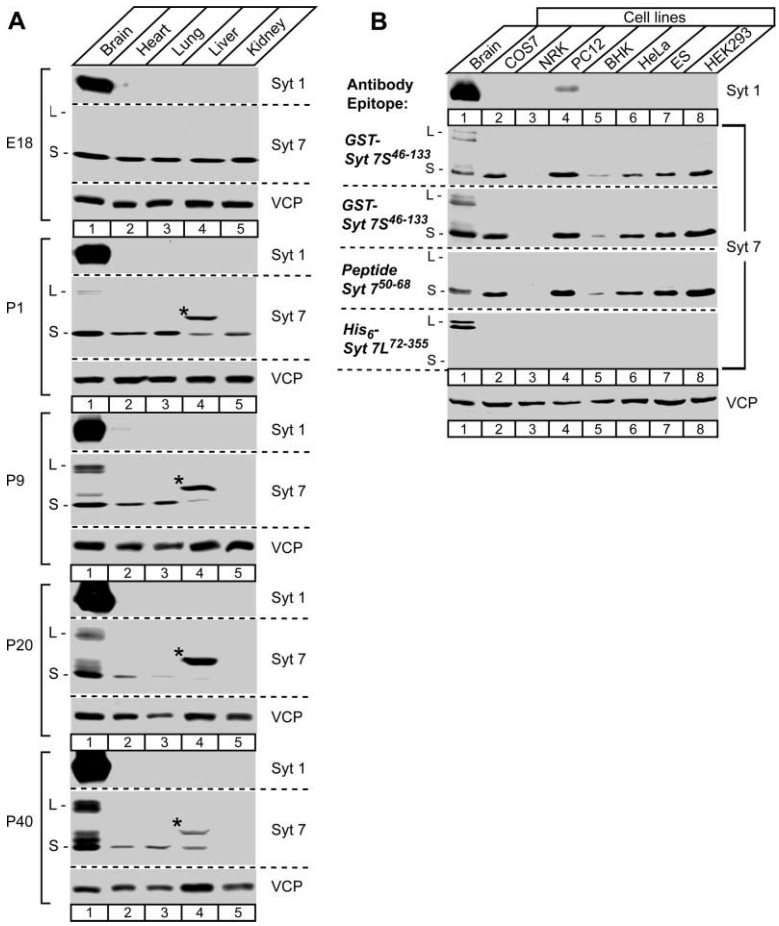

Figure 1. Differential Expression of Synaptotagmins 1 and 7 during Development

(A) Immunoblot analysis of synaptotagmin 1 and 7 in mouse tissues at different stages of development. Equal amounts of protein (80 μg/lane) from the indicated tissues from embryonic day E18 or postnatal days P1 to P40 were examined with antibodies to synaptotagmins 1 (Syt 1) and 7 (Syt 7) and to VCP (vasolin-containing protein, used as a loading control). During development, expression of synaptotagmin 7 becomes increasingly restricted to brain and simultaneously diversified into alternatively spliced low- (L) and high-molecular weight variants (H; see Sugita et al., 2001; asterisks, nonspecific liver band not found with other synaptotagmin 7 antibodies).

(B) Expression of synaptotagmin 7 in various cell lines. Total lysates from brain (lane 1, positive control) and the indicated cells were analyzed by immunoblotting with four independent antibodies to synaptotagmin 7 that were raised against three distinct epitopes. Epitopes are listed on the left; note that the antibody to recombinant His_6-Syt $7L^{72-355}$ is directed to a sequence that is only present in long synaptotagmin 7 splice variants.

natriuretic factor-ECFP, a known component of secretory granules (Figure 2B; see Burke et al., 1997).

It has been suggested that the differential localization of synaptotagmin 1 in neuroendocrine versus nonneuronal cells is due to the selective internalization of synaptotagmin 1 in neuroendocrine but not nonneuronal cells (Jarousse and Kelly, 2001; Dasgupta and Kelly, 2003). To examine this possibility, we produced synaptotagmins 1 and 7 with an N-terminal (extracellular) myc tag and transfected them into PC12 cells. We then incubated the transfected PC12 cells with antibodies to the myc epitope for 1 hr at 0°C or 37°C, washed, fixed, and permeabilized the cells, and visualized the localization of the myc-epitope antibodies by immunofluorescence. At 0°C, we observed efficient cell surface labeling for synaptotagmin 7 but no surface labeling or uptake for synaptotagmin 1 (Figure 2C). At 37°C, we still detected only cell surface labeling for synaptotagmin 7 without internalization but now found intracellular labeling for synaptotagmin 1. Thus, synaptotagmin 7 is resistant to endocytosis even during prolonged incubation at 37°C, while synaptotagmin 1 recycles via the plasma membrane at 37°C but not at 0°C as expected for a membrane trafficking event.

Mapping the Vesicular Targeting Sequence of Synaptotagmin 1

To identify the sequences that mediate the differential localizations of synaptotagmins 1 and 7, we examined whether the C_2 domains of synaptotagmin 1 are essential for internalization. Consistent with previous reports (Jarousse and Kelly, 2001), we found that a truncated synaptotagmin 1 that lacked either the C_2B domain or both the C_2A and the C_2B domain was constitutively localized to the plasma membrane (Figures 3A and 3B). This result raised the possibility that the C_2B domain of synaptotagmin 1 harbors a signal for internalization that is absent from the synaptotagmin 7 C_2B domain, a hypothesis that agrees well with the structural differences between the synaptotagmin 1 and 7 C_2B domains (Fernandez et al., 2001). To test this hypothesis, we constructed chimeric synaptotagmin 1/7 proteins in which the cytoplasmic and noncytoplasmic sequences of synaptotagmins 1 and 7 were exchanged (Figure 3A, chimeras #1 and #2). The chimeras were joined in the middle of the TMR and were examined as EYFP fusion proteins in both PC12 and AtT-20 cells to ensure that the results do not reflect a cell line-specific phenomenon (Figure 3B and Supplemental Figure S1 (http://www.neuron.org/cgi/content/full/41/1/85/DC1).

Surprisingly, we found that transplanting the short N-terminal sequence of synaptotagmin 1 onto synaptotagmin 7 was sufficient to redirect synaptotagmin 7 from the plasma membrane to intracellular vesicles (chimera #1, Figure 3B). Conversely, when we transferred the N-terminal region of synaptotagmin 7 onto the cytoplasmic synaptotagmin 1 sequences, we observed localization to the plasma membrane (chimera #2, Figure

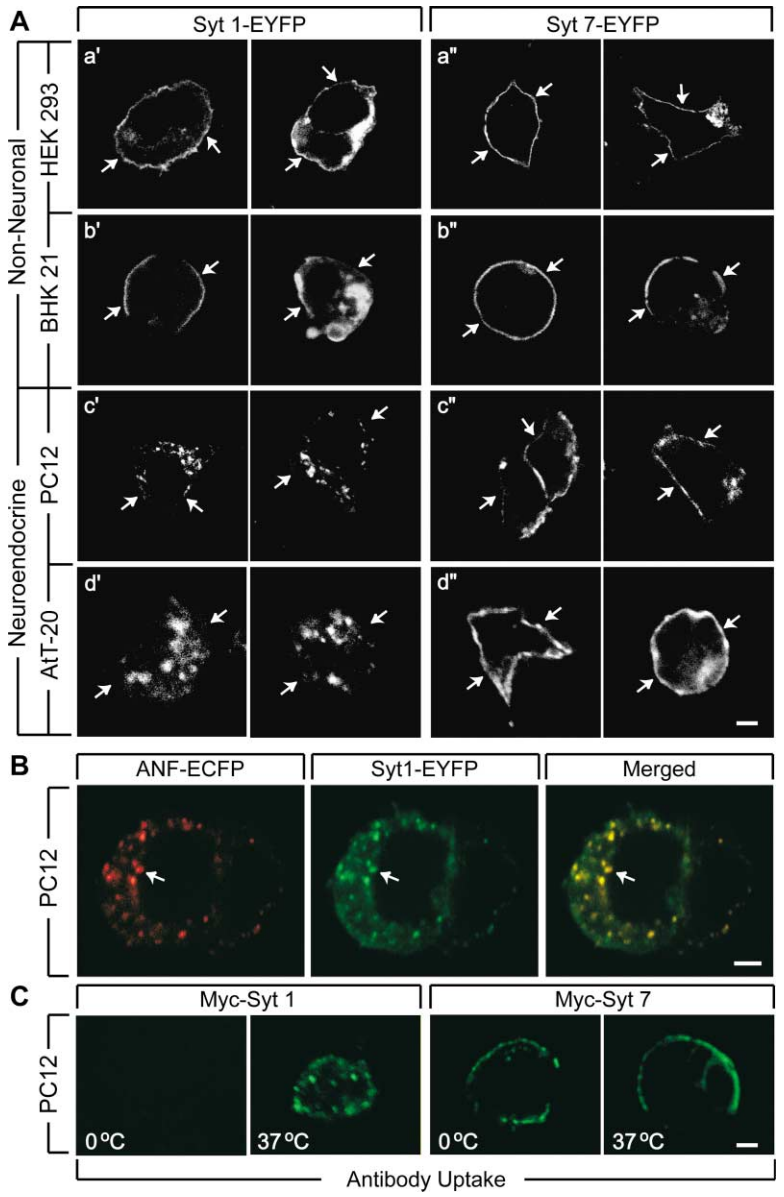

Figure 2. Transfected Synaptotagmin 1 but Not Synaptotagmin 7 Is Internalized into Secretory Vesicles in Neuroendocrine but Not Nonneuronal Cells

(A) Localization of synaptotagmin 1 (Syt 1-EYFP) and 7 (Syt 7-EYFP) expressed as EYFP fusion proteins in nonneuronal HEK293 and BHK21 and neuroendocrine PC12 and AtT-20 cells. Panels exhibit confocal images; arrows point to plasma membrane.

(B) Colocalization of Syt 1-EYFP with a secretory vesicle marker. Pictures show a representative confocal image of a PC12 cell that coexpresses an ECFP fusion protein of atrial natriuretic factor (ANF-ECFP) and Syt1-EYFP.

(C) Internalization of transfected synaptotagmin 1 but not synaptotagmin 7 measured by antibody uptake. PC12 cells were transfected with N-terminally myc-tagged synaptotagmin 1 or 7 and incubated for 60 min at 0°C or 37°C with anti-myc antibodies. Afterward, cells were fixed and permeabilized, and bound antibodies were visualized with Alexa 488-labeled secondary antibodies.

Scale bars in (A), (B), and (C) (equal to 2 μm) apply to all panels.

3B). Thus, although the C_2 domains of synaptotagmin 1 are essential for correct targeting, they can be functionally replaced by the C_2 domains of synaptotagmin 7 in spite of their different localizations. These results indicate that the specificity of the intracellular targeting of synaptotagmins 1 and 7 is mediated by their extracellular sequences, an unexpected result considering the fact that most targeting sequences are cytoplasmic. To ensure that the observed localization was not caused by a construct-specific artifact (e.g., the chimeric TMR) and to narrow down the precise sequence motif that is required for targeting, we produced additional chimeric proteins in which smaller parts of the extracellular sequences of synaptotagmins 1 and 7 were exchanged (Figure 3A). These constructs confirmed that the N-terminal sequence of synaptotagmin 1 directs localization to intracellular vesicles, and restricted the targeting sequence of synaptotagmin 1 down to its N-terminal 29 residues (chimeras #3–#6, Figure 3B).

Intravesicular N-Glycosylation Is Essential for Correct Targeting of Synaptotagmin 1

A major difference between the N-terminal sequences of synaptotagmins 1 and 7 is that the former but not the latter is N-glycosylated (Perin et al., 1991; Sugita et al., 2001). The N-terminal sequence of synaptotagmin 1 contains a single consensus site for N-glycosylation and multiple serines that could potentially be O-glycosylated. Deglycosylation experiments confirmed that brain synaptotagmin 1 is both N- and O-glycosylated (Figure 3C) and that the synaptotagmin 1 EYFP fusion protein is also N-glycosylated (Figure 3D). In contrast, the synaptotagmin 7 EYFP fusion protein is not N-glycosylated but becomes N-glycosylated after the N-terminal sequence of synaptotagmin 1 is inserted (Figure 3D).

To test whether N-glycosylation of synaptotagmin 1 participates in vesicular targeting, we produced a point mutant of synaptotagmin 1 (N24Q) that substitutes asparagine[24] for glutamine, a conservative substitution that

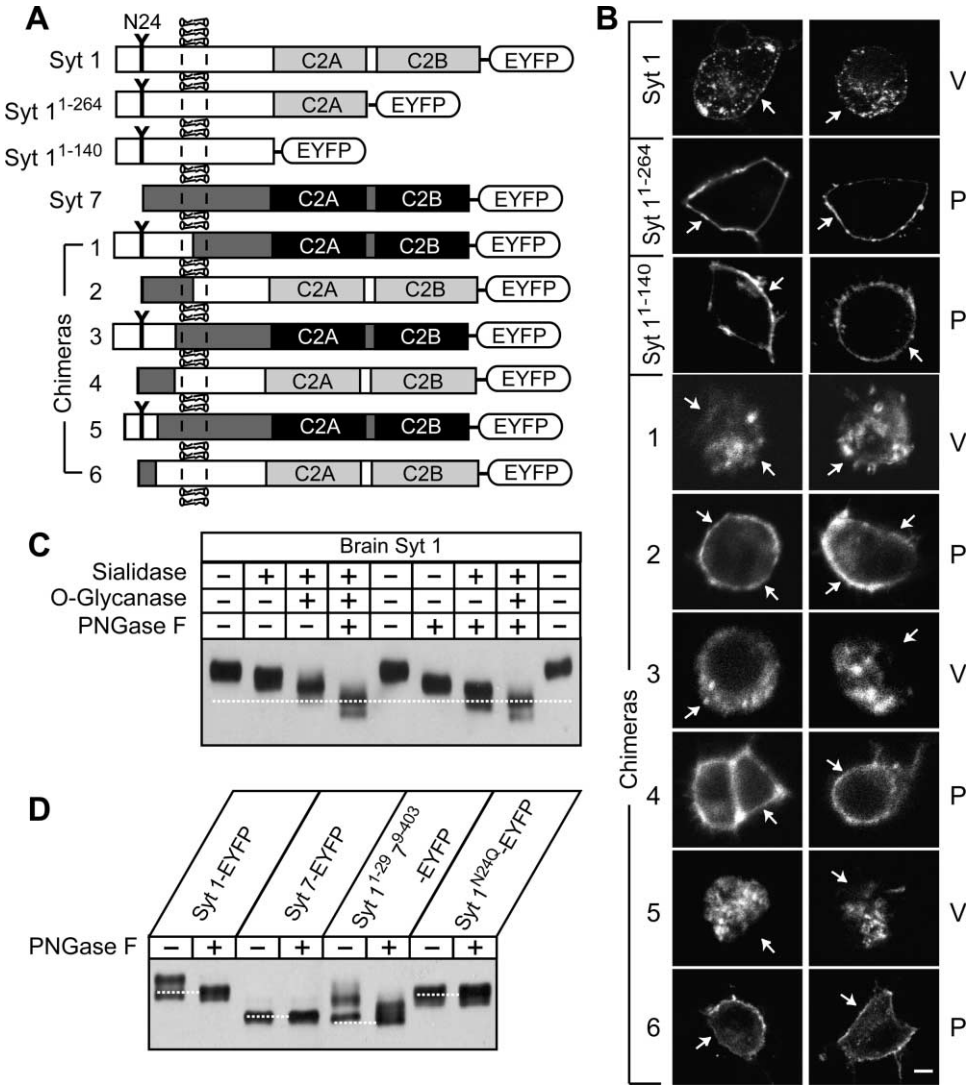

Figure 3. Localization of Truncated and Chimeric Synaptotagmin 1/7 Proteins

(A) Diagram of the structures of synaptotagmins 1 (Syt 1) and 7 (Syt 7), of truncated synaptotagmin 1 variants (Syt 1^{1-264} and Syt 1^{1-140}), and of synaptotagmin 1/7 chimeras. The "Y" on the N-terminal side represents the N-glycosylation site of synaptotagmin 1 at position asparagine[24] (N24). The synaptotagmin 1/7 breakpoints for chimeras #1, #3, and #5 were residues 62/27, 52/18, and 29/9, respectively, and the synaptotagmin 7/1 breakpoints for chimeras #2, #4, and #6 were residues 26/63, 17/53, and 8/30, respectively.

(B) Confocal images of PC12 cells expressing truncated synaptotagmin 1 variants and chimeric synaptotagmin 1/7 proteins fused to EYFP (see panel [A]). Proteins were visualized via their EYFP fluorescence, and letters on the right assign their localizations to two distinctive patterns: plasma membrane (P) versus vesicular (V). The scale bar on the bottom right panel (equal to 2 μm) applies to all images.

(C) N- and O-glycosylation of brain synaptotagmin 1. Proteins from rat brain homogenate were treated with Sialidase, O-Glycanase, and PNGase F in different combinations as indicated to remove neuraminic acid (sialidase), O-linked sugars (O-glycanase), and N-linked sugars (PNGase F). Samples were examined by immunoblotting with a monoclonal antibody to synaptotagmin 1 (CI41.1).

(D) N-glycosylation of transfected synaptotagmins. EYFP fusion proteins of synaptotagmins 1 (Syt 1) and 7 (Syt 7) or chimera #5 (Syt 1^{1-29} 7^{9-403}) and of N24Q mutant synaptotagmin 1(Syt 1^{N24Q}) were expressed in HEK293 cells, incubated without or with PNGase F to remove N-linked sugars, and examined by immunoblotting with a polyclonal antibody to EYFP.

abolishes N-glycosylation (Figure 3D). We transfected wild-type synaptotagmins 1 and 7 and N24Q mutant synaptotagmin 1 as EYFP fusion proteins into PC12 and AtT-20 cells and stained the cells with fluorescent FM4-64 dye as a plasma membrane marker. Confocal microscopy (Figure 4A) revealed that, in both neuroendocrine cells, the N24Q mutation quantitatively redirected synaptotagmin 1 from intracellular vesicles to the plasma membrane. Thus, a single point mutation, N24Q, converted the localization of synaptotagmin 1 to that of synaptotagmin 7.

The plasma membrane localization of the N24Q mutant suggests that intravesicular N-glycosylation of synaptotagmin 1 is essential for endocytosis. To examine this hypothesis by an independent method that does not rely on an EYFP fusion protein, we employed the endocytosis assay described in Figure 2C using N-terminally myc-tagged wild-type and N24Q mutant synap-

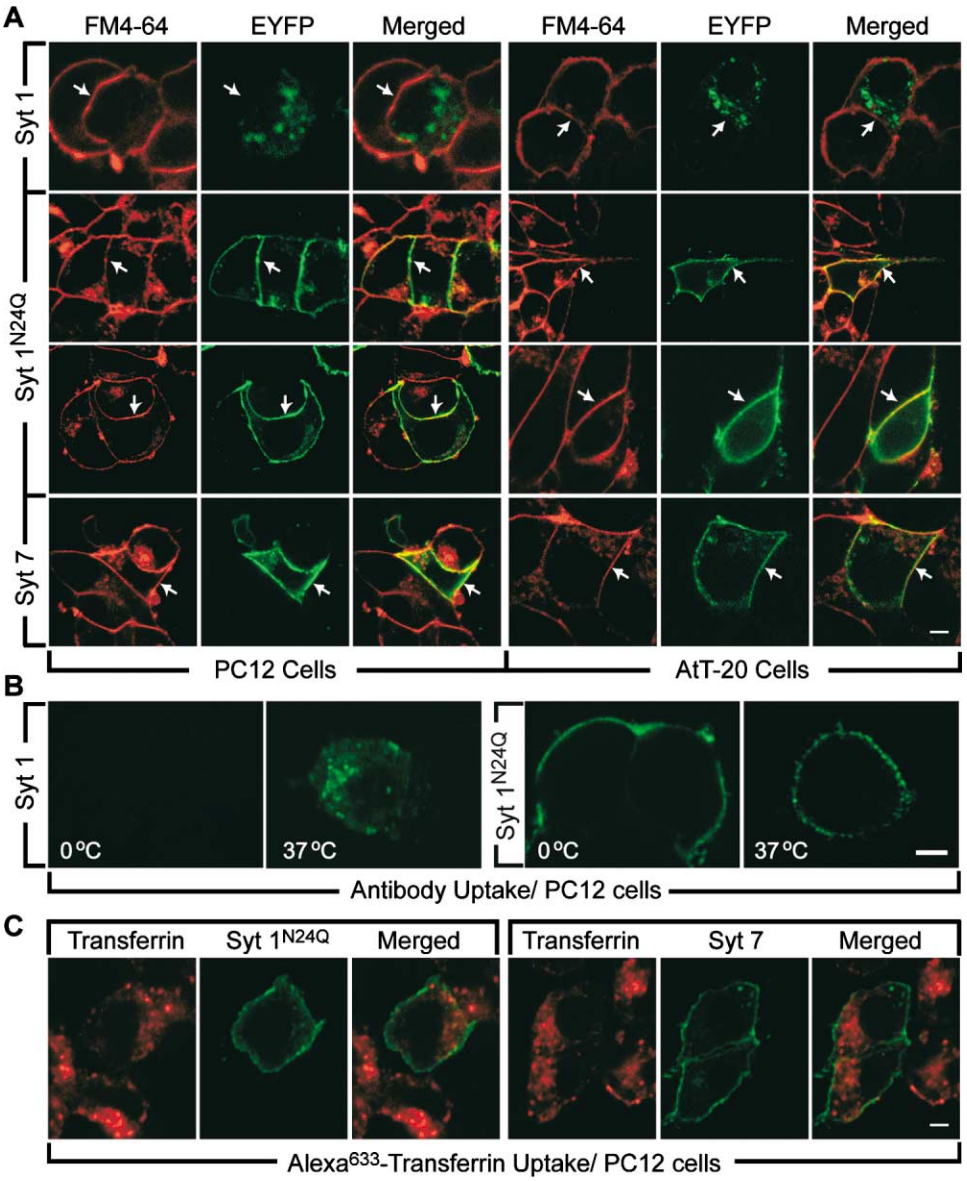

Figure 4. A Point Mutation that Abolishes N-Glycosylation of Synaptotagmin 1 Redirects Synaptotagmin 1 from Secretory Vesicles to the Plasma Membrane

(A) EYFP fusion proteins of wild-type (Syt 1) and N24Q mutant synaptotagmin 1 (Syt 1^{N24Q}) and of wild-type synaptotagmin 7 (Syt 7, used as a control) were expressed by transfection in PC12 cells (left three panels) and AtT-20 cells (right panels). Cells were stained with FM4-64 to label the plasma membrane, and the FM4-64 and EYFP fluorescence were imaged by confocal microscopy. For each panel, signals are merged on the right. For the N24Q mutant of synaptotagmin 1, two separate examples are shown for PC12 and AtT-20 cells to illustrate reproducibility. Immunoblotting of transfected cells showed that the N24Q mutation abolishes N-glycosylation of synaptotagmin 1 (Figure 3D).

(B) The N24Q mutation inhibits synaptotagmin 1 endocytosis. PC12 cells were transfected with N-terminally myc-tagged wild-type or N24Q mutant synaptotagmin 1, incubated with anti-myc tag antibodies as described in Figure 2, and bound antibodies were visualized by indirect immunofluorescence. For quantitations, see Supplemental Figure S2 (http://www.neuron.org/cgi/content/full/41/1/85/DC1).

(C) N24Q mutant synaptotagmin 1 or wild-type synaptotagmin 7 do not impair general endocytosis. Confocal images of PC12 cells that express N24Q mutant synaptotagmin 1 or wild-type synaptotagmin 7 on the cell surface were incubated with Alexa 633-labeled transferrin. The synaptotagmins and internalized transferrin were imaged by fluorescence microscopy to examine whether transferrin-uptake is inhibited by expression of plasma membrane-localized synaptotagmins.

Scale bars in the lower right panels (equal to 2 μm) apply to all panels.

totagmin 1 (Figure 4B). When we applied the antibody to the N-terminal myc tag to the medium, we detected the N24Q mutant synaptotagmin 1 on the cell surface at both 0°C and 37°C. Even after 1 hr of incubation at 37°C, we observed no internalization of the N24Q mutant synaptotagmin 1, whereas wild-type synaptotagmin 1 was transported to intracellular vesicles (Figure 4B). Quantitation of the ratio of intracellular to plasmalemmal fluorescence values using confocal imaging revealed that wild-type synaptotagmin 1 but not N24Q mutant

synaptotagmin 1 was efficiently internalized (Supplemental Figure S2 [http://www.neuron.org/cgi/content/full/41/1/85/DC1]). To exclude the possibility that internalization or lack thereof depended on either the N-terminal myc-epitope or the C-terminal EYFP fusion of synaptotagmin 1, we introduced the N24Q mutation into unmodified synaptotagmin 1 and monitored the cycling of N24Q mutant and wild-type synaptotagmin 1 using a monoclonal antibody to the N-terminal intravesicular sequence of synaptotagmin 1 (Matteoli et al., 1992). The monoclonal antibody only detected surface-exposed synaptotagmin 1 with N24Q mutant but not wild-type synaptotagmin 1 (Supplemental Figure S3). Thus, three types of experiments show that internalization of synaptotagmin 1 in neuroendocrine cells requires intravesicular N-glycosylation.

A potential concern is that N24Q mutant synaptotagmin 1 and/or wild-type synaptotagmin 7 may not normally be plasma membrane proteins but, when overexpressed, suppress endocytosis by an unknown mechanism. To address this concern, we incubated transfected cells with fluorescent transferrin and tested whether expression of N24Q mutant synaptotagmin 1 or wild-type synaptotagmin 7 altered uptake of transferrin (Figure 4C). We detected no difference in transferrin uptake between transfected and adjacent nontransfected cells, suggesting that the cell-surface localization of the N24Q mutant synaptotagmin 1 and wild-type synaptotagmin 7 cannot be explained by a global inhibition of endocytosis.

N-Glycosylation in Combination with Cytoplasmic C_2 Domains Is Sufficient to Mediate Internalization

Is N-glycosylation the signal that mediates internalization of synaptotagmin 1 into secretory vesicles (and of synaptotagmin 7 when it is mutated), or do other features of the intravesicular sequence of synaptotagmin 1 participate? To address this question, we first inserted smaller segments of the synaptotagmin 1 intraluminal sequence into the N-terminal region of synaptotagmin 7 (chimeras #7 and #8, Figure 5A). These chimeras were constructed as N-terminally myc-tagged proteins to allow monitoring of synaptotagmin internalization (see Figure 2C) or as C-terminal EYFP fusion proteins to allow visualization of the steady-state localization of the respective proteins. Synaptotagmin 7 chimeras containing 25 and 18 residues of synaptotagmin 1 were N-glycosylated in transfected 293 cells (Figure 5B) and efficiently internalized in transfected PC12 cells at 37°C but not 0°C (Figure 5C). Furthermore, these chimeras expressed as EYFP fusion proteins were localized to vesicles at steady state (Figure 5D).

We next constructed a chimeric synaptotagmin 7 containing a random 18 residue sequence that contains a similar amino acid composition to the 18 residue synaptotagmin 1 insertion sequence of chimera #8 but shares no sequence homology with synaptotagmin 1 except for the presence of an N-glycosylation site (chimera #9, Figure 5A). The random sequence (SAPSSLAQPAIQ NATDAS) exhibits no sequence homology to a known protein and was solely designed to be rich in short-chain amino acids and include an N-glycosylation consensus sequence near the C terminus. It was inserted into the same position as the synaptotagmin 1 sequence in chimera #8. As a negative control, we substituted glutamine for asparagine in the randomized sequence (chimera #10). In transfected 293 cells, synaptotagmin 7 containing the randomized N-glycosylation sequence was N-glycosylated whereas the NQ mutant of this sequence was not (Figure 5B). We then analyzed both chimeras for vesicular targeting and internalization. Only the N-glycosylated but not the nonglycosylated synaptotagmin 7 chimera was internalized at steady state (Figure 5C) and localized to intracellular vesicles (Figure 5D). These data demonstrate that the exact sequence of the N-terminal region of synaptotagmin 1 is not essential to confer internalization and vesicular localization onto synaptotagmin 7 but that only a functional N-glycosylation site is needed.

Viral Rescue of Synaptic Responses in KO Neurons Lacking Synaptotagmin 1

Is the N-glycosylation of synaptotagmin 1 functionally important for neurotransmitter release? To address this, we first tested whether N-glycosylation is essential for the normal localization of synaptotagmin 1 in neurons. We expressed wild-type and N24Q mutant synaptotagmin 1 (without GFP or an epitope tag) in high-density cultures of hippocampal neurons using recombinant Semliki Forest viruses and selectively visualized synaptotagmin 1 in the plasma membrane by staining fixed, nonpermeabilized neurons with the monoclonal antibody that is directed to the extracellular N terminus of synaptotagmin 1 (Supplemental Figure S4 [http://www.neuron.org/cgi/content/full/41/1/85/DC1]). These experiments showed that >3 times more synaptotagmin 1 was present on the neuronal surface in neurons expressing N24Q mutant synaptotagmin 1 than in neurons expressing wild-type synaptotagmin 1. These results indicate that, in neurons, the internalization of synaptotagmin 1 from the plasma membrane also depends on N-glycosylation, although the widespread overexpression of the virally expressed synaptotagmins does not allow the determination of whether some of the N24Q mutant synaptotagmin 1 additionally "leaked" onto synaptic vesicles, and some of the wild-type synaptotagmin 1 may have lingered on the plasma membrane.

We next investigated whether the differentially localized synaptotagmin 1 variants have distinct effects on Ca^{2+}-triggered synaptic vesicle exocytosis. To examine this question, we employed two control mutants in addition to the N24Q mutant synaptotagmin 1 described above: R233Q mutant synaptotagmin 1 that exhibits a decrease in apparent Ca^{2+} affinity and was characterized in knockin experiments (Fernández-Chacón et al., 2001); and the so-called 6DA mutant synaptotagmin 1 in which Ca^{2+} binding to both C_2 domains is abolished because three critical aspartate residues in the C_2A and the C_2B domain are replaced by alanine residues (hence "6DA"). The R233Q was used as a positive control to validate the rescue system because its phenotype can be predicted from the results of the knockin experiments, and the 6DA mutant was used as a negative control because its inability to bind Ca^{2+} should abolish its function.

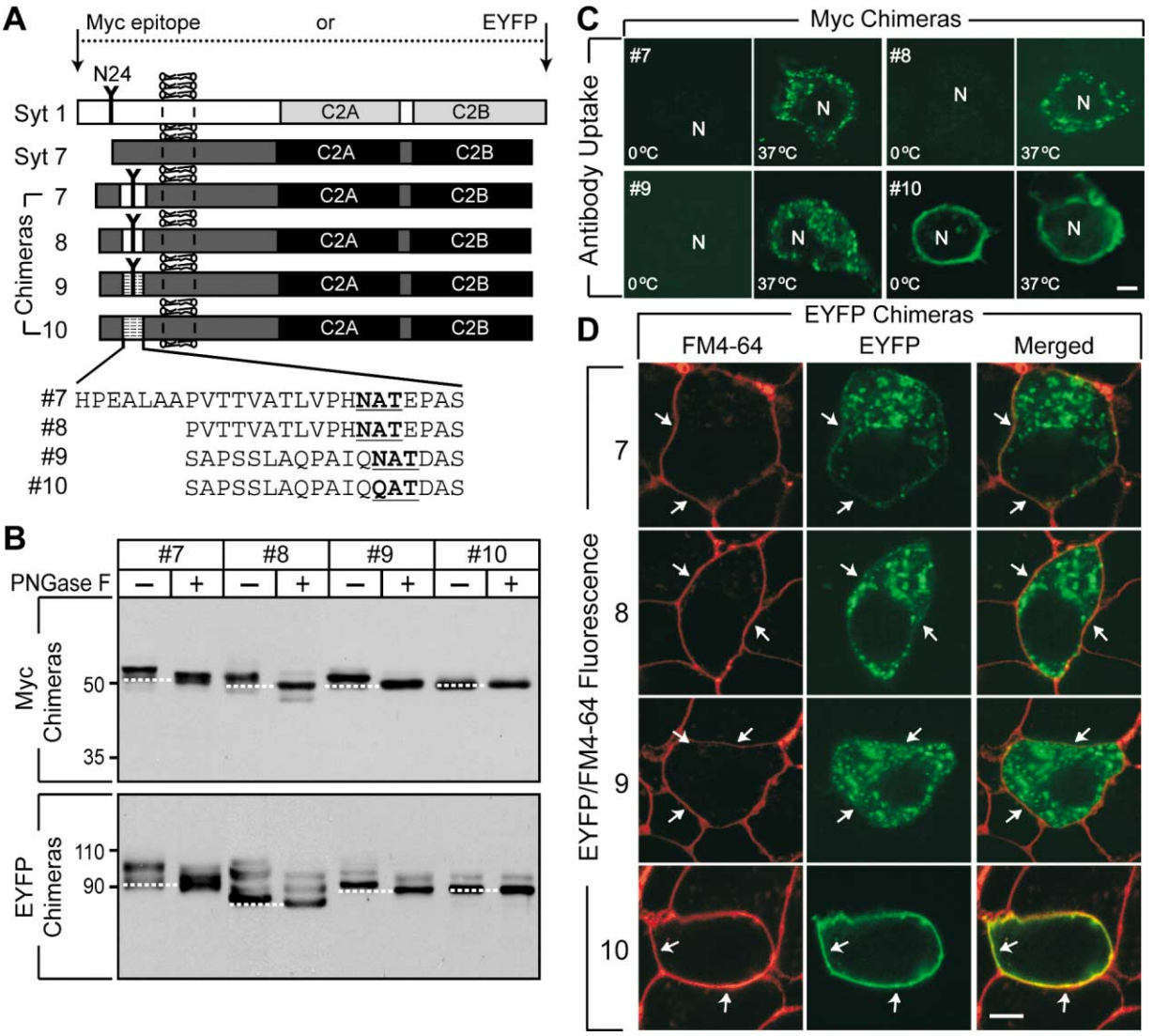

Figure 5. N-Terminal N-Glycosylation Is Sufficient to Redirect Synaptotagmin 7 from the Plasma Membrane into Intracellular Vesicles

(A) Structures of synaptotagmins 1 (Syt 1) and 7 (Syt 7) and of chimeric synaptotagmin 7 proteins. Chimeras #7 and #8 include insertions of 25 and 18 residues from the N-terminal sequence of synaptotagmin 1, while chimeras #9 and #10 include 18 residue insertions in which the synaptotagmin 1 sequence was randomized with (#9) or without retention of the N-glycosylation site (#10). Inserted sequences are shown below the protein diagrams; N-glycosylation consensus sequences are underlined. All proteins were produced and analyzed with either an N-terminal myc epitope tag or a C-terminal EYFP fusion.

(B) N-glycosylation of chimeric synaptotagmin 7 proteins containing 25 and 18 residue insertions.

(C) Confocal images of synaptotagmin internalization experiments. PC12 cells expressing myc-tagged synaptotagmin chimeras #7–#10 were incubated with anti-my antibodies at 0°C or 37°C for 1 hr, fixed, and examined by indirect immunofluorescence for antibody uptake.

(D) Confocal images of PC12 cells expressing various EYFP-tagged chimeric proteins. To clearly identify the plasma membrane in the images (arrows), cells were labeled with FM4-46.

N, nucleus. Scale bars in (C) and (D) (equal to 2 μm) apply to all panels.

We cultured hippocampal neurons from synaptotagmin 1 knockout (KO) mice on isolated microislands of glia cells where neurons form autapses (Geppert et al., 1994), infected the neurons with the recombinant viruses, and recorded evoked synaptic responses in the neurons 15–22 hr after infection (Figure 6A). KO neurons displayed >15-fold smaller peak EPSC amplitudes than wild-type neurons (KO neurons = 0.29 ± 0.08 nA, n = 38; wild-type neurons = 4.5 ± 1 nA, n = 17), and the time course of release was greatly delayed (Figure 6B). Viral expression of wild-type synaptotagmin 1 in KO neurons restored peak EPSC amplitudes (3.1 ± 0.4 nA, n = 57; Figure 6A) and recovered the rapid kinetics of wild-type release (Figure 6B). In contrast, viral expression of the 6DA mutant of synaptotagmin 1 did not rescue either the peak amplitude or the delayed release kinetics (data not shown). Expression of the R233Q mutant, in contrast, fully restored the release kinetics (Figure 6B) but not the amplitude of release (see Figure 7 below), identical to what was described for knockin mice (Fernández-Chacón et al., 2001). In knockin mice, the R233Q mutation decreased the probability of release

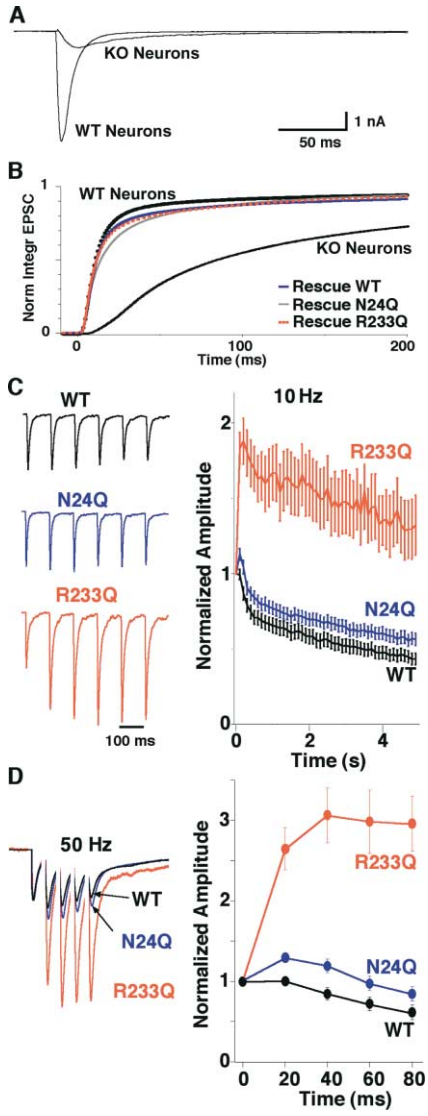

Figure 6. Rescue of the Synaptotagmin 1 KO Phenotype by Viral Expression of Wild-Type and Mutant Synaptotagmin 1

(A) Superimposed representative AMPA receptor-mediated EPSCs from cultured hippocampal wild-type (WT) and synaptotagmin 1 KO (KO) neurons.

(B) Averaged time courses of the normalized integrated charge of EPSCs in response to action potentials. Data are from wild-type (WT, n = 28) and synaptotagmin 1 KO neurons (KO, n = 38) and from synaptotagmin 1 KO neurons infected with Semliki Forest viruses that overexpress wild-type synaptotagmin 1 (WT, n = 35), N24Q mutant synaptotagmin 1 (N24Q, n = 40), and R233Q mutant synaptotagmin 1 (R233Q, n = 23). Time courses were normalized to the total charge integral for 1 s after initiation of the action potential.

(C) Representative traces (left) and summary graphs (right) of synaptic responses recorded in excitatory neurons from synaptotagmin 1 KO mice in response to 10 Hz stimulation. Neurons were infected with recombinant Semliki Forest Virus encoding wild-type synaptotagmin 1 (black trace), synaptotagmin 1^{N24Q} (blue trace), and synaptotagmin 1^{R233Q} (red trace).

(D) Same as in (C), except that neurons were stimulated at 50 Hz. In the summary graphs, synaptic responses were normalized to the first response.

Data in (C) and (D) are means ± SEMs.

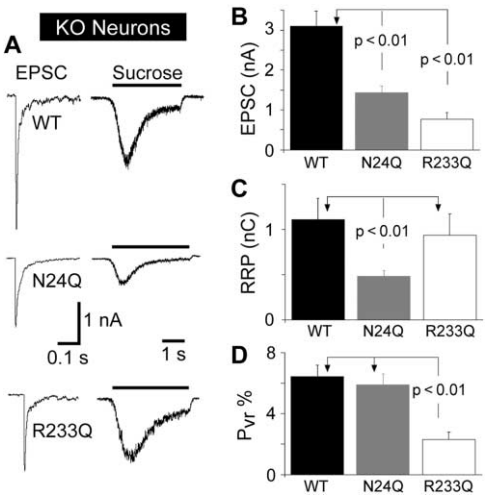

Figure 7. Evoked Responses, Vesicle Pool Sizes, and Vesicular Release Probabilities in Synaptotagmin 1 KO Neurons Expressing Wild-Type, R233Q Mutant, or N24Q Mutant Synaptotagmin 1

(A) Representative evoked EPSCs (left) and sucrose responses (right) from synaptotagmin 1 KO neurons rescued with wild-type synaptotagmin 1 (top, WT), mutant synaptotagmin 1^{N24Q} (middle, N24Q), and mutant synaptotagmin 1^{R233Q} (bottom, R233Q). Rescues were performed by expression with recombinant Semliki Forest viruses.

(B, C, and D) Mean EPSC amplitudes (B), readily releasable vesicle pool size (C), and vesicular release probability (D) from the indicated rescue experiments (wild-type, n = 31; N24Q, n = 36; and R233Q, n = 25). Data shown are means ± SEMs; statistical significance levels are indicated.

which causes facilitation of synaptic responses during repetitive stimulation. This phenotype is also reproduced with virally expressed R233Q mutant synaptotagmin 1 which caused synaptic facilitation during repetitive stimulation at 10 Hz and 50 Hz (Figures 6C and 6D). Together these results validate the rescue approach in spite of the protein overexpression induced by the viral infections.

N24Q Mutant Synaptotagmin 1 Rescues Synaptic Responses but Impairs the Production of Fusion-Competent Vesicles

We next examined whether N24Q mutant synaptotagmin 1 rescues the synaptotagmin 1 KO phenotype (Figures 6 and 7). Similar to the R233Q mutant, the N24Q mutant reversed the delayed release kinetics of the KO phenotype (Figure 6B), but repetitive stimulation elicited no difference in the relative responses of KO neurons expressing either wild-type or N24Q mutant synaptotagmin 1 (Figures 6C and 6D). To test whether the N24Q mutant synaptotagmin 1 was fully functional, we quantified the average size of synaptic responses evoked by action potentials (as a measure of Ca^{2+}-triggered release) and hypertonic sucrose (as a measure of the readily releasable pool). The ratio of these two parameters provides the vesicular release probability (P_{vr}), a sensitive measure of the efficacy of Ca^{2+}-triggered release (Figure 7).

Unexpectedly, we found that N24Q mutant synaptotagmin 1 suppressed the size of both action potential-

induced EPSCs and of sucrose-induced EPSCs by approximately 50% compared to wild-type synaptotagmin 1 (Figures 7B and 7C). In contrast, synapses expressing R233Q mutant synaptotagmin 1 exhibited a selective decrease in Ca^{2+}-evoked release and vesicular release probability but displayed no change in sucrose-induced responses as observed in knockin mice (Fernández-Chacón et al., 2001). Since the vesicular release probability P_{vr} is derived from the ratio of action potential- to sucrose-induced EPSCs, it was decreased in R233Q mutant synapses but normal in synapses expressing N24Q mutant synaptotagmin 1 (Figure 7D), consistent with the unchanged short-term plasticity of these synapses (Figure 6). These results suggest that even though N24Q mutant synaptotagmin 1 can partially rescue the KO phenotype, mislocalization of synaptotagmin 1 due to deficient N-glycosylation in the N24Q mutant has a severe effect on synaptic responses. The synaptic phenotype of the N24Q mutant is surprising because deletion of synaptotagmin 1 itself has no effect on the readily-releasable pool (Geppert et al., 1994, 1997), indicating that mislocalization of synaptotagmin 1 acts as a dominant negative that alters the availability of synaptic vesicles for release.

Effect of Overexpressed Wild-Type and Mutant Synaptotagmin 1 on Neurotransmitter Release in Wild-Type Neurons

If the N24Q mutant synaptotagmin 1 acts as a dominant negative to decrease the size of the readily releasable pool in synaptotagmin KO neurons, it may also alter release in wild-type neurons. However, overexpression experiments of a protein on a wild-type background are difficult to interpret because, for many proteins (e.g., rab3, syntaxin, and synaptophysin), simple overexpression of the wild-type protein on a wild-type background alters synaptic function. Therefore, we compared in wild-type neurons the synaptic effects of three overexpressed proteins: wild-type synaptotagmin 1 to test for a possible synaptotagmin overactivity, N24Q mutant synaptotagmin 1 to probe for a potential dominant-negative role, and 6DA mutant synaptotagmin 1 as an inactive negative control that provides another point of comparison for overexpressed wild-type and N24Q mutant synaptotagmin 1 (Figure 8). As above, all proteins were expressed without a myc or EYFP tag by infecting neurons with recombinant Semliki Forest viruses.

We found that, compared to noninfected neurons, viral expression of the 6DA mutant did not produce significant changes in EPSC amplitude, sucrose response, or vesicular release probability, suggesting that the 6DA mutant is a good control (Figure 8). Overexpression of N24Q mutant synaptotagmin 1 in wild-type neurons caused a substantial decrease in the size of both action potential-induced EPSCs (Figure 8B) and of sucrose-triggered EPSCs (Figure 8C) without a significant change in the release probability (Figure 8C). Thus, N24Q mutant synaptotagmin 1 suppresses synaptic responses in both wild-type and synaptotagmin 1 KO neurons in a similar pattern. Overexpression of wild-type synaptotagmin 1 also caused a synaptic phenotype that, surprisingly, consisted of a large increase (>70%) in release probability and was not observed for the N24Q and 6DA mutant

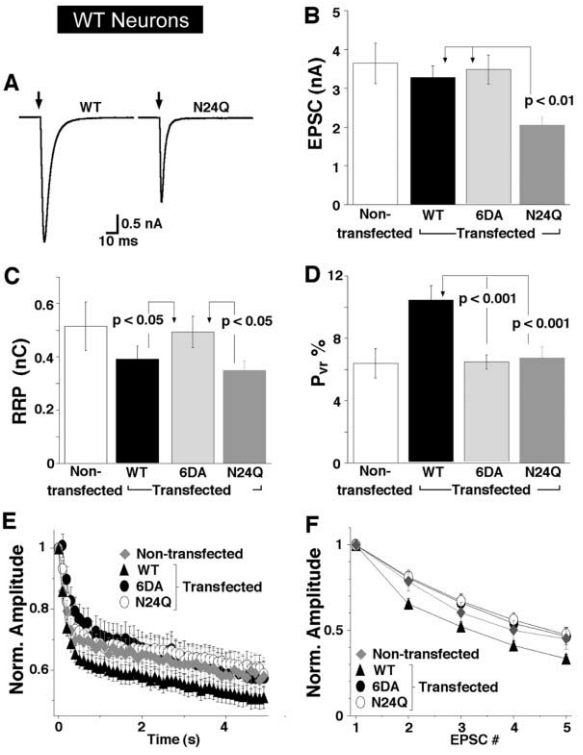

Figure 8. Synaptic Responses in Wild-Type Neurons Expressing Wild-Type, N24Q Mutant, or 6DA Mutant Synaptotagmin 1

(A) Representative EPSC responses from wild-type neurons expressing wild-type (left) or N24Q mutant synaptotagmin 1 (right).

(B, C, and D) Mean EPSC amplitudes (B), readily releasable vesicle pool size (C), and vesicular release probability (D) recorded in naive wild-type neurons (n = 15) or wild-type neurons expressing wild-type synaptotagmin 1 (WT, n = 71), 6DA mutant synaptotagmin 1 (n = 52), or N24Q mutant synaptotagmin 1 (n = 64). Data shown are means ± SEMs; statistical significance levels are indicated.

(E and F) Normalized synaptic responses in naive wild-type neurons or wild-type neurons expressing wild-type or mutant synaptotagmin 1. Responses were recorded during repetitive stimulation at 10 Hz (E) or 50 Hz (F) (noninfected, n = 15–17; WT overexpression, n = 54–77; 6DA, n = 44–48; and N24Q, n = 46–72).

synaptotagmins (Figure 8D). This increase was confirmed in repetitive stimulation experiments which revealed that overexpression of wild-type synaptotagmin 1 induced increased depression, consistent with a higher release probability (Figures 8E and 8F). Viewed together, these experiments establish that mislocalization of synaptotagmin 1 caused by the N24Q mutation results in a change in neurotransmitter release.

Discussion

Synaptotagmins constitute a family of Ca^{2+} binding proteins that are candidate Ca^{2+} sensors in exocytosis. Synaptotagmins share a common domain structure, but at least those synaptotagmins whose localization has been examined in detail (e.g., synaptotagmins 1, 2, 3, and 7; see Perin et al., 1990, 1991; Geppert et al., 1991; Sugita et al., 2001, 2002) are differentially localized. Synaptotagmin 1, a synaptic vesicle protein that regulates the fast component of release (Geppert et al., 1994;

Yoshihara and Littleton, 2002), is arguably the best-studied isoform. The structures of the C_2 domains from synaptotagmin 1 were determined at atomic resolution (Ubach et al., 1998; Fernandez et al., 2001), and Ca^{2+} binding to these C_2 domains was shown to dictate the apparent Ca^{2+} affinity of fast release (Fernández-Chacón et al., 2001). Nevertheless, the mechanisms that target synaptotagmin 1 to synaptic vesicles and segregate it from synaptotagmin 7 (which is also expressed in synapses but present on the plasma membrane) are unclear. On a more fundamental level, even the expression and localizations of synaptotagmins 1 and 7 are not uniformly agreed upon. In addressing these issues, we have made three principal observations: synaptotagmins 1 and 7 exhibit distinct patterns of expression and localization, the N-terminal N-glycosylation site of synaptotagmin 1 is essential for its targeting to synaptic vesicles, and synaptotagmin 1 N-glycosylation is required for its function in exocytosis. These findings uncover an unexpected role for extracellular N-glycosylation in the recycling of synaptic vesicles.

Distinct Expression Patterns of Synaptotagmins 1 and 7

Consistent with previous results, we detected significant levels of synaptotagmin 1 only in brain and neuroendocrine tissues at all developmental stages examined. In contrast, we found that synaptotagmin 7 is abundantly expressed in embryonic mice in all tissues examined (Figure 1A). This pattern changes during postnatal development when the expression of synaptotagmin 7 decreases outside of brain to very low levels but increases in brain. In addition, we observed that synaptotagmin 7 is present in all rapidly proliferating cell lines tested (Figure 1B). Ubiquitously expressed synaptotagmin 7 is synthesized in a single splice variant of 45 kDa in embryonic mice, whereas neuronal synaptotagmin 7 is amplified into a large number of splice variants in adult mice. These results support the notion that synaptotagmin 1 performs a specialized function in neurosecretion, whereas synaptotagmin 7 may have a dual role: a universal function in proliferating cells and a restricted synaptic function in postmitotic neurons. It is possible that both of these functions involve Ca^{2+}-regulated exocytosis, which is also observed in proliferating nonneuronal cells (Coorssen et al., 1996; Ninomiya et al., 1996).

N-Glycosylation of Synaptotagmin 1 Is Required for Its Localization to Secretory Vesicles

Our major finding is that an essential determinant for the endocytosis and vesicular localization of synaptotagmin 1 is its intravesicular N-glycosylation site. The requirement of a noncytoplasmic sequence, embodied in a single amino acid (N^{24}) that is N-glycosylated, for localization of a protein is surprising. Three lines of evidence support the identification of the intravesicular N-glycosylated sequence of synaptotagmin 1 as an essential determinant of synaptotagmin 1 localization:

(1) In transfected neuroendocrine cells (where transfected synaptotagmins 1 and 7 faithfully reproduce the localization of native synaptotagmins [Figure 2]), the localization of chimeric synaptotagmin 1/7 EYFP fusion proteins was dictated by their N-terminal noncytoplasmic sequences (Figure 3). Insertion of only 18 N-terminal residues from synaptotagmin 1—consisting of the N-terminal part of the intraluminal sequence of synaptotagmin 1 without the TMR—into the N terminus of synaptotagmin 7 was sufficient to redirect synaptotagmin 7 from the plasma membrane to intracellular vesicles (chimera #8, Figure 5).

(2) Conversely, abolishing N-glycosylation of synaptotagmin 1 via a single amino acid substitution converted synaptotagmin 1 into a plasma membrane protein in the context of an EYFP fusion protein (Figure 4A), a myc-tagged protein (Figure 4B), and an unmodified protein (Supplemental Figures S3 and S4 [http://www.neuron.org/cgi/content/full/41/1/85/DC1]) in neuroendocrine cells and in neurons. While wild-type synaptotagmin 1 was efficiently endocytosed in transfected PC12 cells, mutant synaptotagmin 1 with a substitution in the N-glycosylation site (N24Q) was retained on the plasma membrane (Figure 4B).

(3) Insertion of a random 18 residue sequence into the N terminus of synaptotagmin 7 induced its internalization and its localization to intracellular vesicles only when this 18 residue sequence was N-glycosylated (Figure 5), demonstrating that the signal for the correct targeting is mediated by the N-glycosylation of the extracellular sequence of synaptotagmin 1 and not by another sequence element.

This evidence was obtained, at least for some of the constructs, in two neuroendocrine cells (PC12 and AtT-20 cells) and in hippocampal neurons, with N-terminal myc-tagged or C-terminal EYFP fusion proteins and without any modification of synaptotagmins. The N-terminal intravesicular sequence of synaptotagmin 1 is not simply a "sorting signal," as the cytoplasmic C_2 domains of synaptotagmin 1 were also required for endocytosis (Figure 3). The fact that the C_2 domains from synaptotagmin 7 could substitute for those of synaptotagmin 1 means that the C_2 domains do not contain an autonomous internalization signal but that an activity of the C_2 domains—possibly the binding of the C_2B domain to AP2 and stonins (Zhang et al., 1994; Martina et al., 2001; Walther et al., 2001)—is essential. This agrees well with previous data suggesting that the C_2B domain of synaptotagmin 1 includes an internalization motif (Jarousse and Kelly, 2001; Dasgupta and Kelly, 2003), although our data argue against the conclusion that this internalization motif functions as an autonomous sorting signal. This difference between our and previous results may be due to the fact that previous experiments utilized fusion proteins in which the N-terminal sequences of synaptotagmins were replaced by the extracellular domains of CD4. Thus, important sequences involved in the localization of synaptotagmins were not included in these studies. The notion that binding of endocytic proteins (stonins and AP2) plays a central role in the requirement for the C_2 domains in synaptotagmin 1 sorting is also supported by the fact that the C_2 domains of synaptotagmin 7 bind to AP2 (Li et al., 1995a), but the role of this binding has not yet been examined.

Synaptotagmin 1 N-Glycosylation Is Functionally Important for Exocytosis

Finally, we found that the N24Q mutant of synaptotagmin 1, when expressed in hippocampal neurons from

synaptotagmin 1 KO mice, rescued the phenotype of these mice but decreased the size of the readily releasable pool by ~50% (Figures 6 and 7). Furthermore, expression of N24Q mutant synaptotagmin 1 in wild-type neurons containing endogenous synaptotagmin 1 also depressed the readily releasable pool by ~40% (Figure 8). The rescue approach was validated by demonstrating that viral expression of wild-type synaptotagmin 1 efficiently reversed the mutant phenotype, whereas viral expression of 6DA mutant synaptotagmin 1 that contains no functional Ca^{2+} binding sites failed to do so. Furthermore, viral expression of R233Q mutant synaptotagmin 1 that we have previously characterized in knockin mice (Fernández-Chacón et al., 2001) produced a precise phenocopy of the R233Q knockin phenotype. Therefore, the N24Q mutant of synaptotagmin 1 appears to act as a dominant-negative protein which alters a function that does not normally require synaptotagmin 1.

These results establish that N24Q mutant and wild-type synaptotagmin 1 are not only targeted to different cellular localizations in neuronal and neuroendocrine cells but also act differently in release. Thus, the intraluminal N-glycosylation site of synaptotagmin 1 is required for its normal function. The correlation between mislocalization and the decrease in the readily releasable pool indicates that the former may cause the latter, especially since all known functions of synaptotagmin 1 depend on its cytoplasmic C_2 domains which are identical between wild-type and N24Q mutant synaptotagmin 1. A plausible interpretation of our results is that synaptotagmin 1, when mislocalized to the plasma membrane, suppresses the readily releasable pool. A possible mechanism for this dominant-negative effect is provided by the constitutive, Ca^{2+}-independent interaction of synaptotagmin 1 with the SNARE complex (Bennett et al., 1992; Rickman and Davletov, 2003; Shin et al., 2003) that may sequester the SNARE complex and cause a decrease in the readily releasable pool.

The electrophysiological results raise a number of questions that we currently cannot answer. Key among these is how N24Q mutant synaptotagmin 1 partially rescues synaptic responses in KO neurons. It is possible that the misdirected N24Q mutant synaptotagmin 1 on the plasma membrane is partially active, i.e., mediates Ca^{2+}-triggered exocytosis but at the same time inhibits the readily releasable pool. Alternatively, the overexpressed N24Q mutant synaptotagmin 1 may "leak" onto synaptic vesicles during endocytosis. Once on the vesicles, the N24Q mutant would act like a normal synaptotagmin 1 since its cytoplasmic sequences are identical to those of wild-type synaptotagmin 1. Another question emerging from the physiological experiments is the mechanism that causes an increase in release probability when wild-type synaptotagmin 1 is overexpressed in wild-type neurons (Figure 8). More synaptotagmin 1 appears to "improve" the synapses, suggesting that the number of synaptotagmin 1 molecules on vesicles contributes to regulating the release probability of a terminal.

Perspectives

Why is intravesicular N-glycosylation required for the correct localization and function of synaptotagmin 1? The mechanistic basis for this requirement may be a neuron-specific factor that couples the N-glycosylated N terminus to other vesicle components. The dependence of the localization of synaptotagmin 1 on its intravesicular N-glycosylation is an unexpected result, since protein targeting normally involves cytoplasmic sequences (see for example Emanuelsson and von Heijne, 2001) and since protein glycosylation generally mediates protein folding or protein-protein interactions (reviewed in Helenius and Aebi, 2001). However, at least in polarized epithelia, N-glycosylation was previously associated with targeting reactions. Apical sorting of some membrane proteins in polarized epithelial cells appears to be mediated by N-glycosylation (Ihrke et al., 2001; Martinez-Maza et al., 2001), whereas other membrane proteins are targeted to apical membranes independent of N-glycosylation (Bravo-Zehnder et al., 2000; Marmorstein et al., 2000). N-glycosylation is probably also not generally responsible for the localization of synaptic vesicle proteins, since many synaptic vesicle proteins are not N-glycosylated, and some vesicle proteins do not even have an intravesicular sequence, suggesting that diverse mechanisms are involved in building a synaptic vesicle.

Experimental Procedures

Vector Construction

A total of 37 expression vectors for transfection or viral expression of various synaptotagmin 1 and 7 proteins were constructed for the present study as described in the Supplemental Data [http://www.neuron.org/cgi/content/full/41/1/85/DC1]. All vectors are based on the rat cDNAs (Perin et al., 1990; Li et al., 1995a).

In Vitro Transcription and Viral Preparation

Linearized SFV plasmids were transcribed in vitro, and the resulting RNA was transfected into BHK21 cells by electroporation (Ashery et al., 1999). One day after transfection, cell culture media containing inactive virus were collected and frozen in aliquots. On the day of infection, one frozen aliquot was thawed and activated by α-chymotrypsin for 30 min. After addition of Aprotinin to inactivate α-chymotrypsin, the viral preparation was used for infection.

Cell Culture and Transfection

PC12 cells were maintained in RPMI medium (Invitrogen) supplemented with 5% horse serum and 10% fetal bovine serum (Invitrogen) in a 37°C incubator with 5% CO_2, while AtT-20 cells and HEK293 cells were kept in DMEM supplemented with 10% fetal bovine serum in a 37°C incubator with 15% CO_2 and 5% CO_2, respectively. BHK21 cells were maintained in MEM (Invitrogen) supplemented with 10% fetal bovine serum, nonessential amino acids and sodium pyruvate in 37°C incubator with 5% CO_2. For imaging, PC12, AtT-20, HEK293, and BHK21 cells were plated on poly-Lysine (Sigma, 1 mg/ml in 0.1 M borate buffer) coated cover glass (18 mm No. 1.5, VWR). One day after plating, cells were transfected with DNA plasmids using Tfx-50 (Promega). For immunoblots, HEK293 cells were plated in 6-well plates and transfected using FuGENE 6 (Roche) one day later. Two days after transfection, cells were collected and lysed in 200 μl of PBS. For immunocytochemistry, AtT-20 cells were plated on poly-Lysine-coated cover glasses. One day after plating, cells were transfected with either pCMV-Syt1 or pCMV-Syt7 using FuGene 6 (Roche). Two days after transfection, cover glasses with cells were washed in PBS and fixed in 4% paraformaldehyde.

Hippocampal CA3-dentate gyrus region was collected from neonatal mice, and dissociated neuronal cultures were prepared as described (Kavalali et al., 1999). Ten days after plating, 10–30 μl of viral preparation of either pSFV-Syt1 or pSFV-Syt1^{N24Q} were added to each well. Six to eight hours later, cells were washed in PBS and

fixed in 4% paraformaldehyde for subsequent immunocytochemistry procedure as described below.

Immunocytochemistry

Fixed AtT-20 cells and hippocampal neurons were blocked in PBS containing 3% goat serum and 3% dry milk for 1 hr at room temperature. Cells were first labeled with a monoclonal antibody against Syt1's N terminus (Cl604.4, 1:500) and Alexa 568-conjugated goat-anti-mouse IgG (1:1000, Molecular Probes). These cover glasses were then blocked again in PBS containing 3% goat serum, 3% dry milk, and 0.1% Tx-100 for 30 min to permeabilize cell membrane. After blocking and permeabilization, cells were probed with a polyclonal antibody against Syt1 C2A (V761, 1:500) and then Alexa 488-conjugated goat-anti-rabbit IgG (1:1000, Molecular Probes). Cover glasses were then mounted on slides and sealed for observation.

Transferrin Uptake

Two days after transfection with pCMV5-Syt1^{N24Q}-EYFP or pCMV5-Syt7-EYFP, PC12 cells on coverslips were first washed twice in modified PBS (PBS^+ = PBS plus 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% BSA, and 5 mM glucose) and incubated in serum-free MEM (GIBCO) for 60 min at 37°C. MEM was then removed, and PBS^+ containing 100 μg/ml Alexa Fluor 633-conjugated transferrin (Molecular Probes) was added to cells. After incubation at 37°C for 60 min, the cells were washed quickly with ice-cold PBS^+, fixed in PBS containing 4% paraformaldehyde, and mounted onto slides for microscopy.

Antibody Uptake

Two days after transfection with various Myc-tagged expression vectors, PC12 cells on coverslips were first washed in saline (120 mM NaCl, 2.5 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, 30 mM Glucose, pH 7.4) plus 3% BSA on ice, then incubated with 2 μg/ml polyclonal anti-Myc (Upstate Biotechnology, NY) at 37°C or on ice. After 1 hr incubation, the cells were washed on ice, fixed using 4% paraformaldehyde, then permeabilized in 0.1% Triton, and finally reacted to Alexa 488 goat-anti-rabbit antibody (Molecular Probes).

Confocal Microscopy

Cover glasses with cells were fixed 48–72 hr after transfection and then mounted on slides for confocal microscopy. Experiments with FM4-64 were done on live cells. In these experiments, cells were first incubated with 100 μl MEM (Invitrogen) containing 16 μM FM 4-64 (Molecular Probes) for 5 min at room temperature and then mounted in an imaging chamber (Warner Instruments). Confocal images were acquired on a BioRad MRC1024 or a Leica TCS2 laser scanning confocal microscope using either a 63× oil (NA1.4) or a 100× oil (NA1.3) objective lens. Confocal images of SFV-infected neurons were quantitated in Metamorph program. Average intensity of each region of interest (ROI) was first corrected for background noise, then signal from anti-N-terminus antibody was normalized to that from anti-C_2A antibody. Data were presented as mean ± SEM.

Electrophysiological Analyses of Cultured Hippocampal Neurons

Individual cultures of hippocampal neurons from all mice in a litter from heterozygous mutant/wild-type synaptotagmin 1 mice were prepared at P0 on microislands of glia cells (preplated in 10% fetal bovine serum) under conditions favoring formation of autapses, and used for experiments after 10–20 days in culture. Before seeding neurons in a density of 500/cm^2, the medium was exchanged to neurobasal medium A (GIBCO) with supplement B27 (GIBCO). Only dots containing single neurons were used. Semliki Forest virus constructs were prepared and used as described (Rosenmund et al., 2002). Rescue experiments using wild-type and mutant synaptotagmin constructs were performed in parallel. In separate experiments, the phenotype of wild-type and synaptotagmin knockout neurons were characterized (Fernández-Chacón et al., 2001). The extracellular recording solution contained (in mM) NaCl, 140; KCl, 2.4; HEPES, 10; glucose, 10; $CaCl_2$, 4; $MgCl_2$, 4; pH 7.3; 300 mOsm. Synaptic transmission was recorded in the whole-cell configuration under voltage-clamp using 1–2 ms depolarizations from −70 mV to 0 mV to induce action potentials. Hypertonic sucrose solutions contained 0.5 M sucrose in addition to the regular external solution. Patch pipette solutions included (in mM) KCl, 125; NaCl, 10; $MgCl_2$, 4.6, ATP-Na_2, 4; creatine phosphate, 15; phosphocreatine kinase (20 U/ml), EGTA, 1; buffer pH 7.3; 300 mOsm. Chemicals were purchased from Sigma.

Miscellaneous Procedures

Polyclonal antibodies to GFP (T3743) were raised against His-tagged recombinant GFP subcloned into BamHI/HindIII site of the pQE9 vector (Qiagen), expressed in BL21(DE3) *E. coli* strain (Stratagene). The recombinant protein was purified on Ni-NTA agarose (Qiagen) according to the manufacturer's protocol and used for immunizations as described (Johnston et al., 1989). For SDS-PAGE and immunoblotting, transfected HEK293 cells were collected and divided into two groups: with one group receiving PNGase F (NEB) treatment and one group receiving mock treatment. Samples were then mixed with SDS sample buffer and boiled. After the samples were resolved by SDS-PAGE, they were electroblotted to Nylon membrane (Amersham). The membrane was probed using polyclonal antibody against GFP (T3743) and horseradish peroxidase-conjugated secondary antibody.

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