BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 326–332 (1996)
ARTICLE NO. 1174

Fatty Acylation of Synaptotagmin in PC12 Cells and Synaptosomes

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Received July 3, 1996

Synaptotagmin I is localized to synaptic vesicles where it functions in the calcium-triggered release of neurotransmitters. Here we demonstrate that synaptotagmin I covalently incorporated [^{3}H]palmitate after metabolic labeling of PC-12 cells and rat brain synaptosomes. Labeling was localized to a tryptic fragment that contains a cluster of cysteine residues adjacent to the molecule's single transmembrane anchor. Neutral hydroxylamine released the [^{3}H]palmitate from this fragment and increased its electrophoretic mobility, demonstrating that acylation occurs at the membrane-proximal cysteine cluster. In addition, hydroxylamine-induced mobility shifts were also apparent for synaptotagmins II and III, suggesting that posttranslational palmitoylation via thioester bonds may be a general modification of all synaptotagmins.

Synaptotagmin I is an integral membrane protein present on synaptic vesicles and large secretory granules (1,2) where it binds calcium-ions (3) and functions in the calcium-triggered release of neurotransmitters (4,5,6,7). In addition, synaptotagmin binds to the clathrin adaptor protein complex AP-2 and may function in endocytosis (8,9). Recently, a number of additional isoforms of synaptotagmin have been identified in rats and mice (10,11,12,13,14,15,40) and in the marine ray (16). Some of these isoforms are expressed in non-neuronal/neuroendocrine tissue, suggesting that this family of proteins may serve as general components in membrane traffic to and from the cell surface.

All members of the synaptotagmin family are integral membrane proteins which span the vesicle membrane once. They possess short amino-terminal intravesicular domains and large cytoplasmic domains that contain two conserved motifs that are designated as C2-domains (2,17). The C2-domains mediate a number of calcium-dependent and calcium-independent interactions of synaptotagmins with putative effector proteins and phospholipids (18,19,20,14,8,21,22,41).

The primary sequence of synaptotagmin I also revealed the presence of a cluster of cysteine residues at the transmembrane-cytoplasmic domain border followed by a highly basic region (Fig. 1). These cysteine residues, flanked by hydrophobic residues on one side and basic residues on the other, are reminiscent of the palmitoylation sites of a number of proteins (23).

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Abbreviations used are: EGTA, ethylene glycol bis(b-aminoethyl ether)-N, N, N′, N′-tetra acetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IP, immunoprecipitation; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; syt, synaptotagmin; syt_{lum}, tryptic fragment of synaptotagmin I containing the luminal domain; syt_{cyt}, tryptic fragment of synaptotagmin I containing the cytoplasmic domain; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-291X/96 $18.00

In addition, it has been noted that many integral membrane proteins are esterified to palmitate at cysteine residues located near the junction of cytoplasmic and membrane spanning regions (24). We therefore examined the possibility that synaptotagmins may be covalently modified by fatty acylation.

MATERIALS AND METHODS

Deacylation of synaptotagmin. Detergent extracts of rat brain synaptosomes were prepared as described previously (20,21). Briefly, synaptosomes were solubilized in immunoprecipitation (IP) buffer (1% Triton TX-100, 10 mM HEPES, pH 7.4 and 250 mM NaCl) at a detergent:protein ratio of 10:1 (wt:wt) for 40 min at 4°C. To selectively cleave synaptotagmin I between residues 111–112 (25,17,19), 750 μl of the detergent extract was treated with 1 μg trypsin for 15 min at room temperature. Digestions were terminated by the addition of a 50-fold excess (w:w) of soybean trypsin inhibitor, 2 mM PMSF, 10 μM aprotinin, 10 μg/ml pepstatin A, 10 μM leupeptin and 5 mM EGTA. To assay for a hydroxylamine-induced shift in electrophoretic mobility, 50 μl of the untreated or trypsin-treated extracts were mixed with 50 μl 2M hydroxylamine, pH 7.0 or 2M Tris, pH 7.0 as a control, for 1 hr at rt. One hundred μl of each sample was precipitated (39) and the pellets were dissolved in 75 μl SDS-PAGE sample buffer (180 mM Tris pH 6.8, 6 % SDS, 30% sucrose) without reducing agents. Samples were boiled for 3 min, subjected to SDS-PAGE on 10 or 12% gels and immunoblotted using mouse monoclonal antibodies directed against the C-terminal luminal domain (antibody 604.1) or the cytoplasmic domain (antibody 4.1) of synaptotagmin, as described previously (19). All SDS-PAGE was carried out using the Bio-Rad Mini Protean Gel II apparatus. Immunoreactivity was visualized using a goat anti-mouse horseradish peroxidase-conjugated secondary antibody and the Enhanced Chemiluminescence reagents (Amersham).

Metabolic labeling. PC-12 cells were grown to confluence on 10 cm dishes in D-MEM with 10% fetal calf serum and 5% horse serum at 37°C with 10% CO_2. Cells were starved for 2 hr in unsupplemented media and subsequently labeled with 2 mCi [^{3}H]palmitic acid (Amersham) in 3.5 ml unsupplemented media for 90 min. Cells were washed four times with PBS, collected and solubilized in 2 ml IP buffer with constant mixing at 4°C for 40 min. Rat cortical synaptosomes (four milligrams) were prepared using a discontinuous Ficoll gradient as described (26) and resuspended in 1 ml of unsupplemented D-MEM. Synaptosomes were labeled with 2 mCi [^{3}H]palmitic acid for 90 min, washed and solubilized with 2 ml of IP buffer, as described above. In some experiments, the synaptosomal detergent extracts (2 mg in 1 ml) were treated with 1 μg trypsin for 20 min at rt. Digestions were terminated by the addition of 1 mg soybean trypsin inhibitor, 2 mM PMSF, 10 μM aprotinin, 10 μg/ml pepstatin A and 10 μM leupeptin.

Immunoprecipitations. All manipulations were carried out at 4°C. Detergent extracts of [^{3}H]palmitic acid labeled PC-12 cells or synaptosomes were centrifuged at 90,000 $\times$ g for 10 min in a TLA 100.3 rotor to remove insoluble material. One ml aliquots of the supernatants containing 1.4–2 mg PC-12 or synaptosomal detergent extracts were incubated with 8 μl of the anti-synaptotagmin I monoclonal antibody 41.1 or the anti-SNAP-25 monoclonal antibody 71.1 (27). One-half ml aliquots of the trypsin treated synaptosomes were incubated with 8 μl of either 41.1 or 604.1 (described above). As controls, duplicate samples lacking the immunoprecipitating antibodies were also included. Following a 90 min incubation, 25 μl Protein G Fast Flow (Pharmacia) was added and incubated for 1 hr. Samples were washed 3 times with IP buffer and once with 10 mM HEPES, pH 7.4. Immune complexes were solubilized by boiling for 3 min in 3$\times$ SDS-PAGE sample buffer without reducing agents. Samples were subjected to SDS-PAGE. Gels were soaked in 10% methanol for 10 min, immersed in Amplify (Amersham) for 15 min, dried and processed for fluorography. Exposures were typically 6 weeks at −70°C. Prior to immersion in Amplify, some gels were soaked in 1 M hydroxylamine or 1 M Tris, pH 7.5 for 4 hours at rt followed by three water washes.

RESULTS AND DISCUSSION

All known isoforms of synaptotagmin contain cysteine residues within or near their membrane spanning-cytoplasmic domain junctions. These cysteine residues, bordered by hydrophobic residues on one side and by positively charged residues on the other side, are reminiscent of palmitoylated regions in other proteins (Fig. 1) (23). Since fatty acylation of proteins can affect their electrophoretic mobility (for example, see ref. 28), we determined whether treatment of synaptosomal detergent extracts with neutral hydroxylamine affected the mobility of synaptotagmin. As shown in Fig. 2, hydroxylamine treatment resulted in a slight increase in the electrophoretic mobilities of synaptotagmins I, II and III. Since thioesters, but not oxyesters or amide bonds, are highly sensitive to neutral hydroxylamine (29), these data indicate that multiple isoforms of synaptotagmin may be acylated at cysteine residues.

In the following experiments we focused on synaptotagmin I, the isoform known to play an essential role in the rapid calcium-triggered release of neurotransmitters from hippocampal

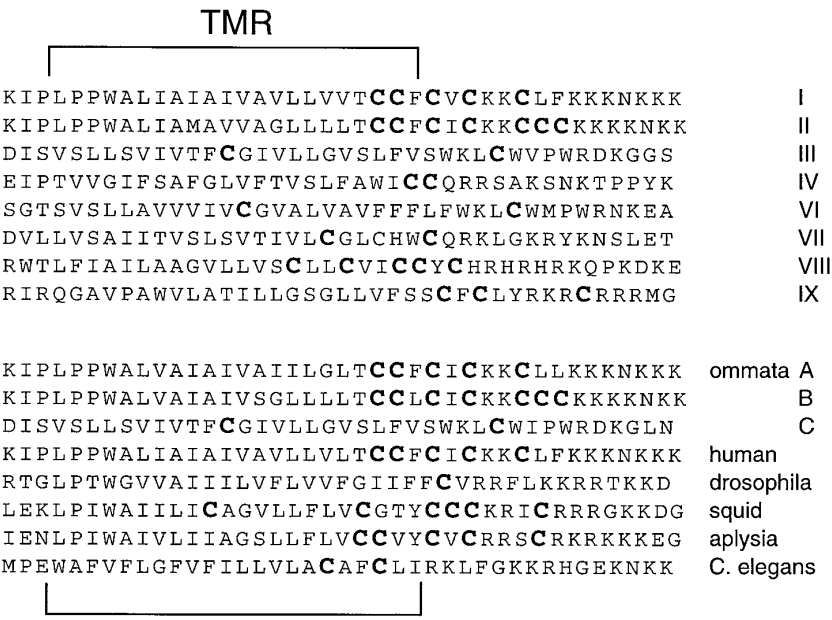

FIG. 1. Amino acid sequences of the transmembrane segment and membrane proximal regions of the synaptotagmin family. Cysteine residues are shown in bold type. The approximate transmembrane region (TMR) is indicated with brackets. In the upper panel, sequences I-IX are from rat, sequence VIII is from mouse. The nomenclature is according to Li *et al.*, (14) with synaptotagmin-5 reported by Hudson and Birnbaum (13) and Craxton and Goedert (15) designated here as synaptotagmin IX. The transmembrane sequence of synaptotagmin V (14) is not yet available.

neurons (10). This isoform contains, in addition to a cluster of five cysteine residues at the membrane-cytoplasmic domain border, a cysteine residue within its second C2-domain (Fig. 3). To determine which of these two cysteine-containing regions of synaptotagmin I was affected by hydroxylamine, we took advantage of a hypersensitive tryptic cleavage site between residues 111–112 (17,19,25). The cysteine-containing domains of synaptotagmin I, syt_{lum} and syt_{cyt}, can therefore be conveniently separated by limited proteolysis and visualized by immunoblotting with the site-specific antibodies 604.1 and 41.1 respectively (Fig. 3 upper panel). As shown in Fig. 3 (lower panel), the hydroxylamine shift was localized to syt_{lum} (recognized by monoclonal antibody 604.1) which contains the transmembrane domain and

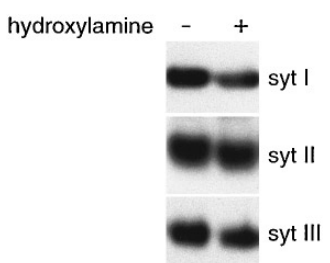

FIG. 2. Effect of hydroxylamine on the electrophoretic mobilities of synaptotagmins I, II and III. Detergent extracts of synaptosomal membranes were treated with 1M hydroxylamine pH 7.0 (+) or, as a control, 1M Tris pH 7.0 (−). Proteins were precipitated, subjected to SDS–PAGE and immunoblot analysis using rabbit antisera specific for either synaptotagmin I, II or III (40), and visualized with the Enhanced Chemiluminescence reagents (Amersham). Hydroxylamine treatment resulted in an increase in the electrophoretic mobilities of all three isoforms of synaptotagmin.

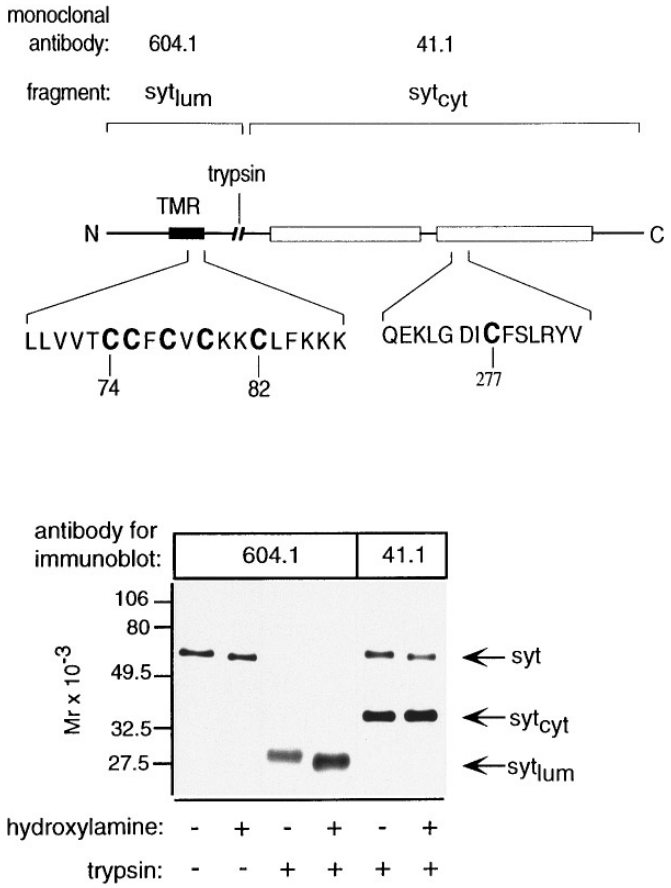

FIG. 3. Effect of hydroxylamine on the electrophoretic mobilities of proteolytic fragments of synaptotagmin I. *Upper panel:* localization of putative acylation sites in synaptotagmin I. Tryptic cleavage results in two distinct proteolytic fragments, designated syt_{lum} and syt_{cyt}, recognized by monoclonal antibodies 604.1 and 41.1, respectively (19). Synaptotagmin contains cysteine residues in each of these two domains and all cysteine residues are indicated in large bold type. The C2-domains are shown as open rectangles. *Lower panel:* Detergent extracts of synaptosomal membranes were treated with 1M hydroxylamine pH 7.0 (+) or, as a control, 1M Tris pH 7.0 (−), with or without prior limited proteolysis with trypsin. The total protein was precipitated and the samples subjected to SDS−PAGE and immunoblot analysis using the monoclonal antibody, 604.1, directed against the luminal domain (syt_{lum}), or 41.1, directed against the cytoplasmic domain (syt_{cyt}). Immune complexes were visualized with the Enhanced Chemiluminescence reagents (Amersham). Hydroxylamine treatment resulted in an increase in the electrophoretic mobility of intact synaptotagmin I as well as syt_{lum}, which contains the cysteine cluster (Fig. 1). No apparent shift in the mobility of syt_{cyt} was observed.

the cysteine cluster. The electrophoretic mobility of syt_{cyt}, which contains a single cysteine residue, was not affected. These data indicate that synaptotagmin I may be acylated at cysteine residues at the membrane-cytoplasmic domain border.

To determine directly whether synaptotagmin I is fatty acylated, we metabolically labeled isolated nerve terminals (synaptosomes) with [^{3}H]palmitic acid. To avoid conversion of the [^{3}H]palmitic acid into other labeled species, labeling times of less than ninety minutes were used. After solubilization with Triton X-100, synaptotagmin I was immunoprecipitated with monoclonal antibody 41.1. As a positive control, the palmitoylated synaptosomal protein SNAP-25 (30), was also immunoprecipitated from parallel samples with monoclonal antibody

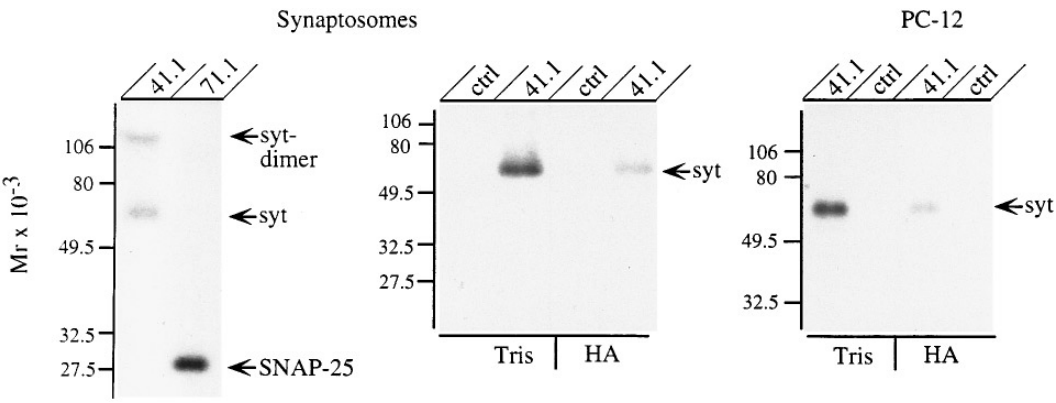

FIG. 4. [^{3}H]palmitic acid labeling of synaptotagmin I in PC-12 cells and in isolated nerve terminals. *Left panel:* Rat cortical synaptosomes were labeled with [^{3}H]palmitic acid and synaptotagmin I was immunoprecipitated with the monoclonal antibody 41.1 as described in Materials and Methods. As a positive control, the palmitoylated protein, SNAP-25 (30) was immunoprecipitated from parallel samples with monoclonal antibody 71.1. Incorporation of [^{3}H]-palmitic acid was determined by subjecting the immunoprecipitates to SDS-PAGE and fluorography. In some experiments we observed synaptotagmin I dimers (syt-dimer) which also incorporated the radiolabel. *Middle and right panels:* Rat cortical synaptosomes or PC-12 cells were labeled with [^{3}H]palmitic acid and synaptotagmin I was immunoprecipitated using monoclonal antibody 41.1, as described in Materials and Methods. As controls (ctrl), parallel samples were processed without added antibody. The immunoprecipitates were subjected to SDS-PAGE under non-reducing conditions. To assay for thioester bonds, gels were soaked for 4 hours in either 1M hydroxylamine (HA) pH 7.5 or 1M Tris pH 7.5, prepared for fluorography and exposed for 6 weeks at −70°C.

71.1, as described in Materials and Methods. The immunoprecipitates were resolved by SDS-PAGE and labeling determined by fluorography. As shown in Fig. 4 (left panel), both SNAP-25 and synaptotagmin I were labeled. We next determined whether the [^{3}H]palmitic acid labeling of synaptotagmin I was sensitive to hydroxylamine. Treatment of the gels with neutral 1M hydroxylamine quantitatively removed the label, indicating that the [^{3}H]palmitate was esterified to cysteine residues (29). Similar results were observed when synaptotagmin was analyzed from [^{3}H]palmitic acid labeled PC12 cells. GAP-43 and SNAP-25 have also been shown to be fatty acylated in isolated neuronal growth cones (30,31). These data indicate that both growth cones as well as mature synaptic terminals contain palmitoyltransferases.

To determine whether syt_{lum} is the sole acylation site of synaptotagmin, [^{3}H]palmitic acid-labeled synaptosomes were treated with trypsin to generate the proteolytic fragments, syt_{lum} and syt_{cyt}, as described in Fig. 3. Immunoblotting with monoclonal antibody 41.1 confirmed that most of the intact synaptotagmin I was converted to the proteolyzed form and that syt_{cyt} was immunoprecipitated with 41.1 (Fig. 5, left panel). Syt_{lum} and syt_{cyt} were immunoprecipitated using monoclonal antibodies 41.1 and 604.1 and incorporation of the label was analyzed by SDS-PAGE and fluorography, as described in Fig. 3. As shown in Fig. 5 (right panel), syt_{cyt} in the 41.1 immunoprecipitates did not incorporate label whereas the minor fraction of intact synaptotagmin I was labeled. These data demonstrate that this fragment is not fatty acylated. In contrast, intact synaptotagmin, as well as syt_{lum} immunoprecipitated by monoclonal antibody 604.1 was labeled.

Palmitoylation was first reported as a post-translational modification of an integral membrane protein, the vesicular stomatitis virus glycoprotein (32). A number of additional viral and cellular integral membrane proteins have been subsequently shown to be palmitoylated (24,33,34,35,36,37). While palmitoylation can clearly serve to attach soluble proteins to membranes, the role of palmitoylation of integral membrane proteins remains less well established. However, palmitoylation has been reported to regulate the rate of internalization of lutenizing

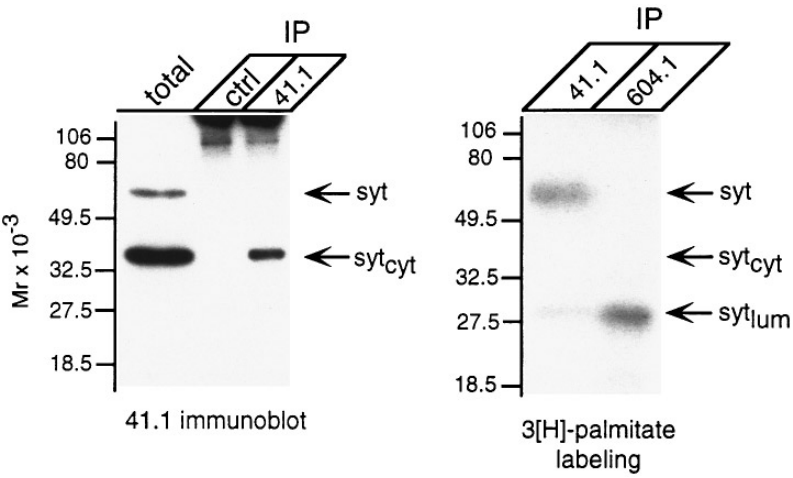

FIG. 5. The membrane proximal cysteine residues of synaptotagmin I are fatty acylated. *Left Panel:* Limited proteolysis of synaptotagmin I and immunoprecipitation of syt_{cyt}. Synaptosomes were metabolically labeled with [^{3}H]palmitic acid, solubilized with Triton X-100 and subjected to limited proteolysis with trypsin as described in Materials and Methods. Syt_{lum} and syt_{cyt} were immunoprecipitated with monoclonal antibodies 604.1 and 41.1, respectively, as described in Materials and Methods. To ensure that the syt_{cyt} fragment was generated and subsequently immunoprecipitated, the total extract (total) and 41.1 immunoprecipitate (IP) were subjected to SDS-PAGE and immunoblotted using antibody 41.1 and [^{125}I]protein A as described (19). Syt_{cyt} is the predominant form in both the total extract as well as in the immunoprecipitate. *Right Panel:* Specific [^{3}H]palmitic acid labeling of syt_{lum}. The luminal and cytoplasmic proteolytic fragments, syt_{lum} and syt_{cyt}, were immunoprecipitated with monoclonal antibodies 604.1 and 41.1, respectively, as described above. The immunoprecipitates were subjected to SDS-PAGE and fluorography as described in Materials and Methods. The results are shown in right panel; using either antibody, the radiolabel was incorporated into the intact protein (syt). Immunoprecipitation with 604.1 also demonstrates that syt_{lum}, containing the transmembrane region and the cysteine cluster (Fig. 1), incorporated the label. No detectable label was observed in syt_{cyt} immunoprecipitated with 41.1, even though this fragment was the predominant species in the immunoprecipitate (see left panel).

hormone/choriogonadotropin (37) and of the transferrin receptor (35). These findings are of particular interest since synaptotagmin binds the clathrin adaptor protein complex AP-2 (8). In addition, synaptotagmin deficient C. elegans appear to exhibit defects in synaptic vesicle endocytosis (9). Therefore, it is tempting to speculate that palmitoylation of synaptotagmin may regulate synaptic vesicle membrane traffic in nerve terminals. It is also notable that membrane transport through the Golgi appartus requires palmitoyl-CoA (38). While the mechanism by which palmitoyl-CoA stimulates membrane transport is not known, these findings suggest that palmitylation of proteins may be important for their function in this pathway. In this light, it will be of interest to determine whether palmitoyl-CoA is required for membrane traffic in the nerve terminal and whether there are synaptotagmin isoforms which function in intracellular membrane transport.

ACKNOWLEDGMENTS

We thank Christiana Walch-Solimena for helpful discussions and Julia Avery for critically reading this manuscript.

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