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Palmitoyl protein thioesterase (PPT) localizes into synaptosomes and synaptic vesicles in neurons: implications for infantile neuronal ceroid lipofuscinosis (INCL)

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A deficiency of palmitoyl protein thioesterase (PPT) leads to the neurodegenerative disease infantile neuronal ceroid lipofuscinosis (INCL), which is characterized by an almost complete loss of cortical neurons. PPT expressed in COS-1 cells is recognized by the mannose-6-phosphate receptor (M6PR) and is routed to lysosome, but a substantial fraction of PPT is secreted. We have here determined the neuronal localization of PPT by confocal microscopy, cryo-immunoelectron microscopy and cell fractionation. In mouse primary neurons and brain tissue, PPT is localized in synaptosomes and synaptic vesicles but not in lysosomes. Furthermore, in polarized epithelial Caco-2 cells, PPT is localized exclusively to the basolateral site, in contrast to the classical lysosomal enzyme, aspartylglucosaminidase (AGA), which is localized in the apical site. The current data imply that PPT has a role outside the lysosomes in the brain and may be associated with synaptic functioning. This finding opens a new route to study the neuropathological events associated with INCL.

INTRODUCTION

Neuronal ceroid lipofuscinoses (NCLs) are common progressive encephalopathies of childhood. These severe brain diseases are divided into four autosomal recessive subtypes, infantile, late infantile, juvenile and adult, based on the age of onset, clinical picture and ultrastructural findings (1,2). Currently, eight different gene loci (*CLN1–CLN8*) have been implicated in NCL and, among these, the infantile form (INCL) is the most severe. INCL is characterized by normal development to the age of 6–12 months, followed by visual deterioration leading to blindness, mental retardation, ataxia and myoclonia. The electroencephalograms of victims of INCL are isoelectric by the age of 3 years. Neuropathologically, INCL is characterized by an almost complete loss of cortical neurons and the cortex is filled by fibrillary astrocytic gliosis. Typical granular storage material with osmiophilic deposits (GROD) is found throughout the brain (3). Interestingly, the major proteins in the storage material are saposins A and D (4), whereas in all other NCL subtypes accumulation of subunit C of the mitochondrial ATP synthase is detected.

Mutations in the *CLN1* gene encoding palmitoyl protein thioesterase (PPT; EC 3.1.2.22) have been found to cause INCL (5). Recently it has been demonstrated that mutations in *CLN1* also account for patients with later-onset NCL who share the same ultrastructure (GRODS) (6–8). PPT is involved in the degradation of lipid-modified proteins and removes fatty acids from cysteine residues *in vitro* (9,10). PPT has recently been crystallized and this has provided a structural basis for the different INCL phenotypes caused by PPT deficiency (11). Lysosomal targeting of PPT has been demonstrated in several different non-neuronal cell lines, and in COS-1 cells it has been shown to be mediated by mannose-6-phosphate receptor (M6PR) (12–14). Two other NCL proteins, i.e. tripeptidyl peptidase 1 (TPP1) and CLN3, defective in late infantile and juvenile NCL, respectively, have been demonstrated to localize in lysosomes (15,16) but also other cellular localizations have been suggested for CLN3 (17). Recent evidence has shown that the CLN8 protein, involved in the NCL disorder Northern epilepsy, is found in the endoplasmic reticulum (ER) and the ER–Golgi intermediate compartment (18). Thus, the NCLs do not seem to be solely lysosomal storage disorders, but also appear to be connected with the trafficking route from the ER to the endocytic pathway.

In mouse and rat brain, PPT mRNA expression is developmentally regulated, increasing gradually during the maturation of the central nervous system. Developmental expression of PPT parallels synaptogenesis, and reaches a maximum in young adulthood. In the adult mouse brain, the signal expression is most intensive in the cerebral cortex, hippocampal pyramidal cells and hypothalamus (19,20). In a study of human embryonic brains, the expression of PPT also increased during brain

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development (21). In mouse brain as well as in neuronal cultures, immunohistochemical analyses have demonstrated that PPT localizes in the neuritic shafts and nerve terminals and colocalizes well with the synaptic markers, synaptophysin and SV2 (20,22). Together, these data imply an important role for PPT in prenatal neuronal development, as well as with postnatal maintenance of neuron function. Furthermore, the distribution of PPT in mouse brain and neuronal cells has indicated that PPT may function outside the lysosomes.

In order to obtain greater insight into the cortical events associated with PPT deficiency, we have here investigated in detail the subcellular localization of PPT using both peripheral non-polarized and polarized cells and neuronal cells. The data imply that in neurons PPT is not localized in the lysosomes, but rather in synaptosomes and synaptic vesicles.

RESULTS

PPT is not lysosomal in mouse brain or cultured primary neurons

Immunofluorescence and fractionation. We have previously shown that both endogenous PPT in mouse brain (20) as well as adenovirus-mediated human PPT in cultured neurons (22) can be found in neuritic shafts and nerve terminals and colocalizes with the synaptic proteins, synaptophysin and synaptic vesicle marker SV2. Here we replicated the immunofluorescence localization studies with recombinant palmitoyl protein thioesterase–Semliki Forest virus (PPT–SFV) infection and PPT was detected with a polyclonal glutathione *S*-transferase (GST)–PPT antibody. Also in this case PPT was targeted to neurites and nerve terminals. Furthermore, PPT was colocalized with SV2 but surprisingly not with the lysosomal LAMP1 staining (Fig. 1). To ensure that no mislocalization occurred due to overexpression, we also studied endogenous mouse PPT by fractionation of mouse brain tissue. We isolated the lysosomal fraction, synaptosomes and synaptic vesicles and compared the amount of PPT in different fractions with that found in total brain cell lysate on western blots. Using a PPT-specific polyclonal peptide antibody, 336/13 PPT was seen as a 37 kDa band in the total brain lysate and was slightly enriched in the synaptosomes. PPT was also present in the synaptic vesicle fraction with a similar intensity as seen in the synaptosomes (Fig 2A). A clear enrichment of the 38 kDa synaptic vesicle-specific protein, synaptophysin, could be demonstrated from synaptosomes to synaptic vesicles indicating the purification of the vesicle fraction (Fig. 2B). Furthermore, we compared the western blot analysis of PPT and the lysosome/late endosome protein LAMP1 (antibody ID4B) in total brain lysate and lysosomes. The lysosomal marker was clearly present in the lysosome fraction, but no PPT could be demonstrated in lysosomes (Fig. 2).

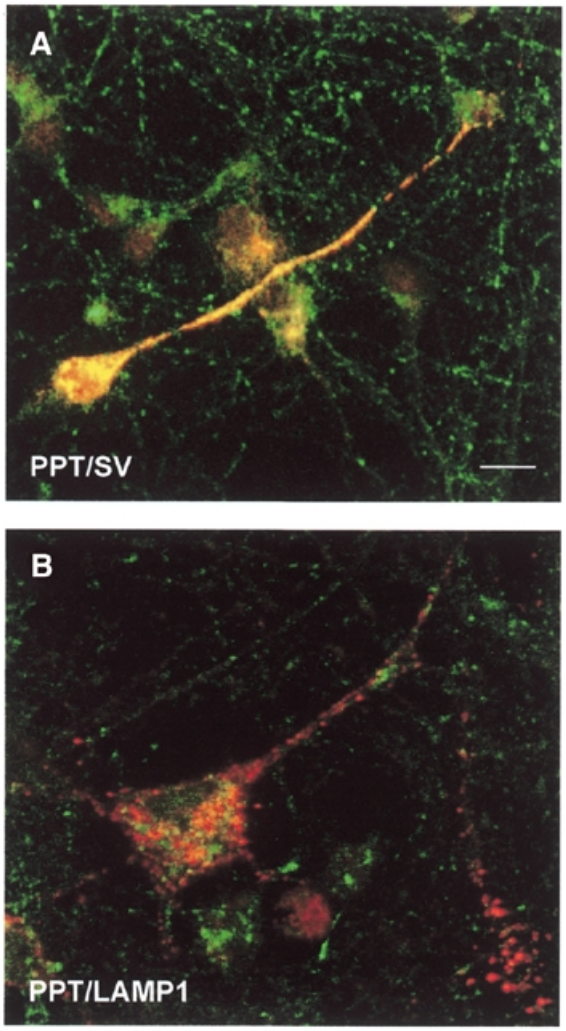

Figure 1. Immunofluorescence analysis of SFV-mediated PPT in primary mouse cortical neurons. Neurons infected with PPT–SFV were double-stained for PPT using the polyclonal GST–PPT antibody (red), synaptic vesicles (**A**) using the monoclonal SV2 antibody (green) and lysosomal membrane protein (**B**) using the monoclonal LAMP1 antibody (green). Bar, 10 μm. Co-localization is shown in yellow.

Immunoelectron microscopy

To further confirm these results we used cryoimmunoelectron microscopy and double-immunolabelled isolated synaptosomes with antibodies to PPT and synaptophysin. We detected both proteins inside the synaptosomes (Fig. 3C). In order to make a comparison, we also immunolabelled PPT from baby hamster kidney (BHK) cells infected with PPT–SFV and Chinese hamster ovary (CHO) cells stably expressing recombinant PPT. In both of these cells, PPT was found inside the lysosomes and late endosomes together with the lysosome/late endosome marker LGP120/LAMP1 (Fig. 3A and B).

These data indicate that, even though PPT has been classified as a lysosomal enzyme due to its lysosomal localization in several different non-neuronal cells, it is specifically not found in lysosomes in mouse brain and is instead present in synaptosomes and synaptic vesicles.

Expression and uptake of PPT in polarized Caco-2 cells

In order to compare the subcellular localization of PPT with classified lysosomal enzymes, we expressed PPT and aspartylglucosaminidase (AGA) (23) in polarized epithelial Caco-2 cells. The SFV-mediated expression of PPT and AGA was followed by immunofluorescence analysis. PPT and AGA were shown to be transported to opposite poles in Caco-2 cells. PPT could be detected exclusively on the basolateral site,

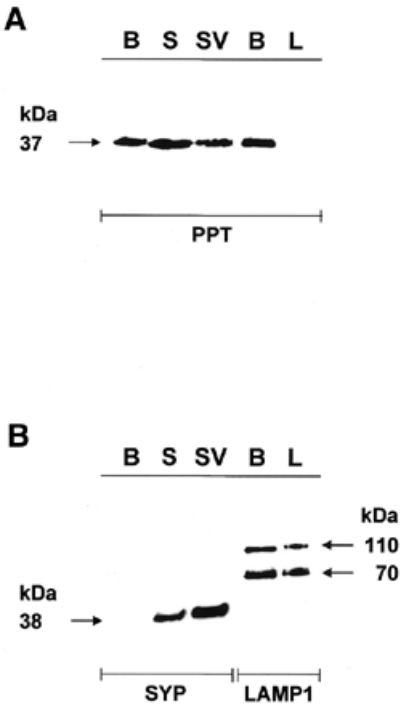

Figure 2. Immunoblotting of PPT in mouse brain fractions: total brain lysate (B), synaptosomes (S), synaptic vesicles (SV) and lysosomes (L) stained with 336/13 polyclonal PPT antibody (**A**) and stained for synaptophysin (SYP) and LAMP1 antibodies (**B**).

whereas AGA was detected on the apical site of Caco-2 cells (Fig. 4A and D). We also let Caco-2 cells endocytose recombinant PPT or AGA from either their basolateral or their apical sites. After endocytosis, AGA was again detected in the apical and PPT in the basolateral sites independently of the site of their endocytosis (Fig. 4B and E). Thus, after endocytosis, AGA was evidently transported from the basolateral to the apical site, and PPT from the apical to the basolateral site.

DISCUSSION

Two earlier studies in COS-1 cells have shown that PPT is a lysosomal enzyme, phosphorylated on mannose residues and targeted through M6PR (13,14). However, in comparison with lysosomal enzymes in general, PPT is relatively unstable in lysosomes. In overexpression studies, a significant amount of the enzyme is secreted, and the secreted enzyme seems to be more stable than that located lysosomally (13,24). Furthermore, it has been shown that PPT has both acidic and neutral pH optima, depending on the palmitoylated substrate used *in vitro* (10,14). This suggests a possible function for PPT also outside the lysosome. In peripheral cells, PPT is intracellularly localized strictly to lysosomes, as previously confirmed by immunofluorescence analysis and cell fractionation studies (13,14). In this study, we confirm the lysosomal localization of PPT protein in CHO and BHK cells using cryoimmunoelectron microscopy.

In order to elucidate the disease mechanism in INCL, it is essential to unravel the cellular routing of PPT in neurons. Our previous results with recombinant adenoviruses showed that PPT is transported to neurites and growth cones in cultured primary cortical neurons (22). Here we used SFV-mediated expression of PPT to demonstrate that the same phenomenon can be observed with another expression system. In immunofluorescence, the overexpressed PPT colocalized with synaptic vesicle marker SV2 but not with the lysosomal marker LAMP1. We also fractionated mouse brain tissue and showed that endogenous PPT is found in a slightly enriched form in synaptosomes. PPT is present in synaptic vesicles but it is not more enriched from the synaptosomal fraction than synaptophysin. In contrast, PPT was not detected in the lysosome fraction of mouse brain. These interesting findings suggest that PPT may have functions other than the lysosomal function, and in the brain it is especially targeted to the synapses and synaptic vesicles.

PPT is a depalmitating enzyme and, especially in the nervous system, there are a large number of palmitoylated proteins, including ion channels, neurotransmitter receptors, signal transduction components, cell-adhesion molecules and many unidentified neuronal and glial proteins. Palmitoylation occurs both at the plasma membrane and in the early secretory pathway and, based on a requirement for palmitoyl-CoA, it has also been suggested to play a role in vesicular transport (25). For example, the palmitoylation of the synaptosomal associated protein of 25 kDa (SNAP-25) is necessary for vesicular transport of proteins, and dynamic fatty acylation of SNAP-25 has been observed in neuronal growth cones and synaptosomes (26,27). It has been proposed that reversible fatty acylation may regulate soluble *N*-ethylmaleimide-sensitive factor attachment protein complex formation during exocytosis (28). Furthermore, targeting of the neuronal growth-associated protein of 43 kDa (GAP-43) is mediated by palmitoylation (29). The importance of palmitoylation indicates a specific role for PPT in the central nervous system and it has recently been demonstrated that PPT can depalmitate several neurospecific peptides *in vitro* (10). Furthermore, PPT overexpression has been shown to lead to reduced membrane association of the palmitoylated GAP-43 (30).

The palmitoylation of both SNAP-25 and GAP-43 has been shown to be dependent on an intact secretory pathway, indicating that they are palmitoylated during their transport through the secretory pathway (31). This is different from many palmitoylated proteins, which have been demonstrated to be directly targeted and palmitoylated in the plasma membrane (32). Furthermore, it has been proposed that multiple palmitoylation and depalmitoylation of SNAP-25 and GAP-43 could serve as a sorting signal for packaging into specific transport vesicles, preceding fast axonal transport in nerve terminals (26). The characterization of the cellular and molecular mechanisms underlying the transport and palmitoylation of neuronal proteins in the exocytic pathway is still somewhat unclear. However, it seems that PPT is localized in the correct compartments of the exocytic pathway to serve as a depalmitoylating enzyme for some specific neuronal protein(s).

Our previous data have indicated polarized localization for PPT in mouse cortical neurons (22). The exact mechanism for this is currently unknown, but in this study we compared the localization of a classical lysosomal enzyme, AGA, and PPT in polarized epithelial Caco-2 cells. Previously we have shown that in cortical neurons AGA was distributed to the cell soma and neuronal processes, but no co-localization with synaptophysin could be detected (33). Here we compared the expression

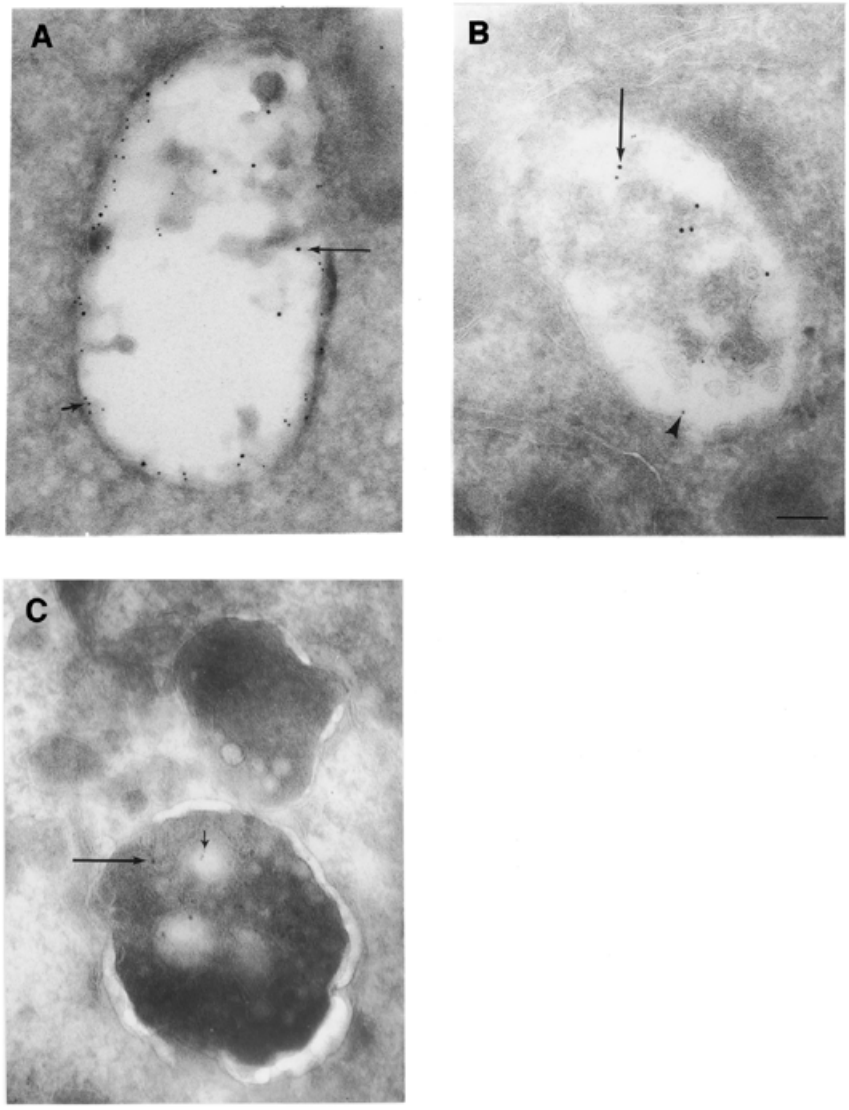

Figure 3. Cryoimmunoelectron microscopy of PPT. BHK cells infected with PPT–SFV (**A**), CHO cells stably transfected with PPT (**B**) and mouse brain synaptosomes (**C**) were double-immunolabelled for GST–PPT polyclonal antibody (10 nm Gold) (long arrows) and LGP120 (5 nm Gold) (A), LAMP1 (5 nm Gold) (B) and synaptophysin (5 nm Gold) (C). Bar, 100 nm; magnification, ×40 000.

pattern of AGA and PPT also in peripheral polarized Caco-2 cells and found that PPT was expressed on the basolateral site and AGA on the apical site of Caco-2 cells. This further confirms that PPT has a different cellular routing than classical soluble lysosomal enzymes. In 1990, Dotti and Simons (34) proposed that neurons and polarized epithelial cells share common mechanisms of protein targeting, with the apical type equivalent to axonal targeting and the basolateral type equivalent to somatodendritic targeting. However, the results of several later studies were inconsistent with this proposal (35,36) and the same phenomenon is observed with AGA and PPT.

The previous data indicated that PPT would merely function in the degradation of fatty-acylated proteins in the lysosomes (9,13). Our current finding implicates that PPT may have a crucial role outside the lysosomes in the brain and may be associated with the synaptic functioning. It is now essential to study further the neuronal routing of PPT to be able to identify the cellular compartments where there are interactions between PPT and palmitoylated neuronal proteins. Only after this has been done can the basic disease mechanism which leads to a severe loss of cortical neurons be unravelled.

MATERIALS AND METHODS

Cell culture

BHK (ATCC CCL-10) cells were cultured in Glasgow minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 10% tryptose phosphate broth, 20 mM HEPES pH 7.0, 100 IU/ml penicillin and 100 g/ml streptomycin.

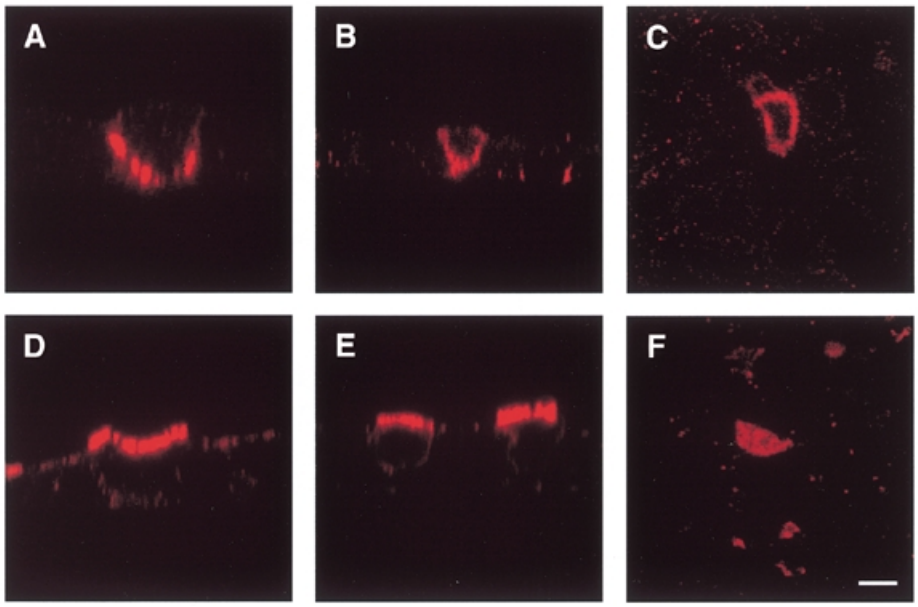

Figure 4. Localization of intracellular and endocytosed PPT and AGA in polarized epithelial cells. Caco-2 cells infected with PPT–SFV (**A**) or AGA–SFV (**D**) and Caco-2 cells fed with recombinant PPT–media (**B**) or AGA–media (**E**) illustrated in *xz*-scores. The *xz*-sections were taken in 1 µm steps through the cells at 90° to *xy*-sections. The endocytosed PPT (**C**) and AGA (**F**) protein in Caco-2 cells are also shown in the *xy*-score. Bar, 10 µm.

Caco-2 cells (ATCC HTB-37) were cultured on polycarbonate filters (Transwell 12 mm ø, pore size 0.4 µm; Costar) in Eagle's MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acids, 100 IU/ml penicillin and 100 g/ml streptomycin.

Cortical neurons were prepared as described by Heinonen *et al.* (22). Briefly, embryos of 14.5–15.5 days post-fertilization were removed and the telencephalic structures were separated. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Life Technologies) supplemented with antibiotics, 0.5 mM L-glutamine, 2.5 µM glutamic acid, 10 mM HEPES pH 7.4 and 1× B-27 (Gibco BRL).

Generation of stably transfected cell line

CHO dhFr⁻ cells (ATCC CRL-9096), cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS, 2 mM L-glutamine, 100 µM hypoxanthine, 10 µM thymidine, 100 IU/ml penicillin and 100 g/ml streptomycin, were transfected with 5 µg of PPT–pSV poly and 0.5 µg of pMMTVdhfr using the Lipofectin transfection reagent (Gibco BRL). The transformants were selected in IMDM without thymidine and hypoxanthine. Resistant cell clones were screened by immunofluorescence and western blot using polyclonal PPT antibodies.

Preparation of the recombinant SFV infections

The coding regions of the PPT and AGA cDNA were cloned to the *Bam*HI site of the pSFV vector as previously described (33). The recombinant PPT–SFV and the AGA–SFV were prepared as described by Olkkonen *et al.* (37). Primary cortical neurons had been maintained in culture for 7–14 days and Caco-2 cells for 8 days prior to infection, and the infection was continued for 1 h at 37°C and 5% CO_2, then the virus solution was drained and the incubation was continued for 6–14 h.

Immunofluorescence

Immunofluorescence staining was performed as previously described (33). For double immunostaining, polyclonal PPT peptide antibody 336/13, a rabbit antiserum against a synthetic peptide corresponding to amino acids 103–119 of human PPT protein (20), was combined with mouse anti-synaptic vesicles (SV2), developed by Kathleen M. Buckley (Harvard Medical School, Boston, MA) and rat anti-lysosomal membrane protein (LAMP1, ID4B), developed by Thomas August (Johns Hopkins University, Baltimore, MD) which were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences at the University of Iowa. Secondary antibody incubation was performed with rhodamine red-conjugated goat anti-rabbit IgG and fluorescein (FITC)-conjugated goat anti-mouse/rat IgG (1:200; Jackson Immuno-Research). The coverslips were mounted in GelMount (Biomeda) and viewed with a Leica confocal microscope (Leica Microscopy and Scientific Instruments Group).

Immunoelectron microscopy

Immunoelectron microscopy was performed according to the method of Tokuyasu (38). Cells were fixed in 4% paraformaldehyde (PFA) + 0.05% glutaraldehyde (Sigma) in 0.2 M PIPES (Calbiochem) pH 7.2 for 2 h at room temperature. The cells were embedded in 10% gelatin, immersed in 15% polyvinylpyrrolidone + 1.7 M sucrose in 0.1 M PIPES for 2 h at 4°C and mounted on specimen holders for cryosectioning. Ultrathin cryosections were cut and selected on 2.3 M sucrose in PIPES. The grids were double immunolabelled using polyclonal

GST–PPT antibody with goat anti-rabbit IgG Gold 10 nm (British BioCell International) and the following monoclonal antibodies: mouse synaptophysin (Dako), mouse anti-LGP120 (a gift from Jean Gruenberg, Department of Biochemistry, Switzerland) and mouse anti-LAMP1 (UH1; developed by B.L. Granger and S. Uthayakumar, obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa), with goat anti-mouse IgG Gold 5 nm (British BioCell International). To obtain PPT protein for immunization, the PPT cDNA was subcloned into the pGEX-2T vector and the PPT polypeptides were expressed in *Escherichia coli* strain B21 as a GST fusion protein and purified from the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (13). Immunolabelled sections were contrasted with uranyl acetate, embedded into uranyl-methylcellulose and viewed with a Jeol 1200 EX with a voltage of 60 kV.

Tissue fractionation

Isolation of synaptosomes of mouse brains was performed as described (39). Briefly, 10 mouse forebrains were gently homogenized in 60 ml of SolA buffer (320 mM sucrose, 5 mM Na-HEPES/HCl, pH 7.4) with twelve strokes at 800 r.p.m. in a glass-Teflon homogenizer. The homogenate was centrifuged (3000 r.p.m., SS34, for 5 min). The resulting postnuclear supernatant was centrifuged twice at 10 000 r.p.m. (SS34, for 20 min) and the crude synaptosomal fraction was resuspended in SolA buffer. This sample was layered on top of a discontinuous sucrose gradient of 1.2 M/1.0 M/0.85 M. After centrifugation for 2 h at 24 600 r.p.m. in an SW28 rotor (Beckman) the synaptosomes were collected at the 1.0 M/1.2 M interphase and resuspended in Krebs solution.

Synaptic vesicles were isolated as described (40,41). Ten mouse cortices were homogenized in 50 ml of buffered sucrose (320 mM sucrose/4 mM Na-HEPES/HCl, pH 7.3). The crude synaptic vesicle fraction obtained by differential centrifugation was resuspended in 40 mM sucrose and layered on top of a linear continuous sucrose gradient of 800 mM/50 mM. Gradients were centrifuged for 5 h at 22 600 r.p.m. in an SW28 rotor (Beckman). Gradients were collected in 1 ml fractions and controlled by refractometry. Corresponding fractions of two identical gradients were pooled to a final volume of 2 ml and 1.5 ml of each fraction containing vesicles was resuspended in 10 mM Na-HEPES/HCl, pH 7.4, 150 mM NaCl to dilute the sucrose and sedimented by centrifugation for 13 h at 47 400 r.p.m. in a 70Ti rotor (Beckman). The pellets were dissolved in Krebs solution and the purity of the fractions was assessed with organelle markers by immunoblotting.

Lysosomes were isolated from mouse brain (42). Briefly, two mouse forebrains were homogenized in 2 ml of 250 mM sucrose, 3 mM imidazole–HCl pH 7.4 (buffer A), and passaged through a 21 G needle. The homogenate was centrifuged and the postnuclear supernatant was layered on top of a step gradient of the following composition (from bottom to top): 2 M sucrose, Percoll (Amersham) in buffer A with the density adjusted to 1.090 g/ml and Percoll in buffer A with a density of 1.075 g/ml. Centrifugation was performed in a Beckman 65.1 Vti rotor for 40 min at 21 200 r.p.m. and stopped without braking. Fractions of 0.5 ml were collected and assayed for purity with organelle markers by immunoblotting.

Western blot

The protease inhibitors (Complete; Boehringer), 8.5 M urea (Promega), 100 mM dithiothreitol (DTT) (Sigma) and Laemmli gel loading buffer (43) were added to the samples of total brain lysates, lysosomes, synaptosomes and synaptic vesicles. The samples were heated for 5 min at >65°C and electrophoresed on freshly made 14% SDS–polyacrylamide vertical slab gels, the 5% stacking gel including 8.5 M urea. The proteins were transferred to nitrocellulose filters (Hybond ECL; Amersham). The filters were blocked overnight at +4°C in TBST containing 5% non-fat dry milk, incubated for 1 h with polyclonal 336/13 PPT antibody (20), anti-synaptophysin (Dako) or ID4B-antibody (LAMP1, developed by Thomas August, Johns Hopkins University, Baltimore, MD and obtained from the Developmental Studies Hybridoma Bank) and for 1 h with horseradish peroxidase-conjugated swine anti-rabbit IgG or goat anti-mouse/rat IgG (Dako). To visualize the bands' enhanced chemiluminescence, 1.25 mM luminol (Fluka) and 0.2 mM *p*-coumaric acid (Sigma) were used.

Uptake of extracellular PPT in polarized Caco-2 cells

Caco-2 cells were infected with PPT–SFV or AGA–SFV virus for 16 h and the media containing the secreted recombinant PPT and AGA was collected and transferred to uninfected cells for 24 h. The cells were fixed with 4% PFA and the filters were immunostained on both sides of the filter with polyclonal GST–PPT or polyclonal native AGA (44) antibodies, followed with rhodamine red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch).

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