The Niemann-Pick C1 protein in recycling endosomes of presynaptic nerve terminals

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Abstract Niemann-Pick type C (NPC) disease is a fatal, neurodegenerative disorder caused in 95% of cases by loss of function of NPC1, a ubiquitous endosomal transmembrane protein. A biochemical hallmark of NPC deficiency is cholesterol accumulation in the endocytic pathway. Although cholesterol trafficking defects are observed in all cell types, neurons are the most vulnerable to NPC1 deficiency, suggesting a specialized function for NPC1 in neurons. We investigated the subcellular localization of NPC1 in neurons to gain insight into the mechanism of action of NPC1 in neuronal metabolism. We show that NPC1 is abundant in axons of sympathetic neurons and is present in recycling endosomes in presynaptic nerve terminals. NPC1 deficiency causes morphological and biochemical changes in the presynaptic nerve terminal. Synaptic vesicles from $Npc1^{-/-}$ mice have normal cholesterol content but altered protein composition. ■ We propose that NPC1 plays a previously unrecognized role in the presynaptic nerve terminal and that NPC1 deficiency at this site might contribute to the progressive neurological impairment in NPC disease.—Karten, B., R. B. Campenot, D. E. Vance, and J. E. Vance. **The Niemann-Pick C1 protein in recycling endosomes of presynaptic nerve terminals.** *J. Lipid Res.* 2006. 47: 504–514.

Supplementary key words cholesterol • synaptosomes • synaptic vesicles

Niemann-Pick type C (NPC) disease is a fatal, neurological disease caused in 95% of cases by loss of function of the NPC1 protein. The remaining 5% of individuals with NPC disease, in whom disease progression is clinically indistinguishable from that caused by mutations in the *NPC1* gene, carry a mutation in another gene, *NPC2* (also called *HE1*). The biochemical hallmarks of NPC disease have been described extensively and include an accumulation of unesterified cholesterol and other lipids in the endocytic pathway as well as a reduced ability to respond to changes in cellular cholesterol levels (reviewed in 1–3). Even though cholesterol homeostasis is disturbed in pe-

ripheral tissues, leading to the enlargement of the liver and spleen, the brain is the only organ in which progressive cell death ensues. Consequently, the most devastating symptoms of NPC disease are neurological, including progressive ataxia, cataplexy, supranuclear gaze palsy, and impairment of the swallowing reflex (4, 5).

The NPC1 protein is a ubiquitously expressed, transmembrane glycoprotein that localizes to late endosomes/lysosomes but also cycles through the Golgi apparatus (6, 7). Although the exact function of the NPC1 protein is still unknown, the presence of a sterol-sensing domain in its sequence (8, 9) and the accumulation of cholesterol in NPC1-deficient cells (10, 11) indicate a role for NPC1 in cholesterol trafficking. NPC2 is a small, soluble, cholesterol binding protein (12–14) that is widely distributed and, like NPC1, is located in late endosomes/lysosomes (reviewed in 15). The function of NPC2 also remains elusive. The classical model of NPC1 function, as described in cultured NPC1-deficient fibroblasts, involves a role in the egress of endocytosed, lipoprotein-derived cholesterol from lysosomes (10, 11, 16). Additionally, in NPC1-deficient cells, the distribution of cholesterol that has been synthesized endogenously is delayed after its reendocytosis from the plasma membrane (17). The trafficking of gangliosides and other glycosphingolipids is also perturbed (18–20). In NPC1-deficient brains, cholesterol is sequestered in late endosomes of glia (21) as well as in cell bodies of neurons (22), and impaired anterograde transport of cholesterol in neurons leads to a decrease in the amount of cholesterol in axons (22, 23).

In light of the global alterations of lipid trafficking in all cells lacking functional NPC1, a question arises: Why are neurons so vulnerable to NPC1 deficiency? In previous work, we have shown that NPC1 is present not only in neuronal cell bodies, where late endosomes and lysosomes mainly reside (24, 25), but also is abundant in distal axons

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Abbreviations: GFP, green fluorescent protein; LAMP, lysosome-associated membrane protein; NPC, Niemann-Pick type C; VAMP, vesicle-associated membrane protein.

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(23). Furthermore, vesicles containing a chimeric protein of NPC1 fused to green fluorescent protein (GFP) move bidirectionally along distal axons of mouse sympathetic neurons (26). The role of NPC1 in distal axons is not clear because axons are not generally thought to be a major site of lysosomal, degradative pathways (24, 25). We hypothesized, therefore, that the presence of NPC1 in axons indicates a neuron-specific function in axons in addition to the classical role of NPC1 in lipid trafficking from late endosomes/lysosomes.

Our experiments demonstrate that NPC1 is present in recycling endosomes in the presynaptic nerve ending and that a lack of NPC1 results in morphological and biochemical changes in the presynaptic terminal.

MATERIALS AND METHODS

Materials

L15 medium was purchased from Invitrogen (Burlington, Ontario, Canada). Other cell culture materials were from BD Biosciences (Bedford, MA). Mouse 2.5S nerve growth factor was purchased from Alomone Laboratories, Ltd. (Jerusalem, Israel). Rat serum was provided by the University of Alberta Animal Services. Protein A-Sepharose was from Sigma. Polyacrylamide gel electrophoresis and immunoblotting supplies were from Bio-Rad (Mississauga, Ontario, Canada). Rabbit anti-human NPC1 polyclonal antibodies, a generous gift from Dr. D. Ory (Washington University, St. Louis, MO), were raised against a peptide consisting of amino acids 1,261–1,272 of human NPC1 and recognize rat and mouse NPC1 in immunoblotting experiments. The polyclonal rabbit anti-mouse NPC1 antibodies used for immunocytochemical studies were raised against a peptide consisting of amino acids 1,254–1,273 of NPC1 and were provided by Dr. W. S. Garver (University of Arizona, Tucson, AZ). The polyclonal rabbit anti-mouse NPC2 antibodies were a gift from Dr. P. Lobel (Rutgers University, Piscataway, NJ). The mouse anti-rat synaptophysin antibodies were from Sigma (Sigma S5768), and the mouse anti-rat vesicle-associated membrane protein-2 (VAMP2) antibodies were from Synaptic Systems (Goettingen, Germany). Mouse anti-human porin 31HL antibodies were from Calbiochem (Mississauga, Ontario, Canada). Rat polyclonal anti-mouse lysosome-associated membrane protein-1 (LAMP1) antibodies were from BD Biosciences PharMingen (Mississauga, Ontario, Canada). Alexa Fluor 488-labeled goat anti-rabbit IgG and Texas Red-labeled goat anti-mouse IgG were from Molecular Probes (Eugene, OR). Adenoviruses containing a NPC1-GFP cDNA (26) were provided by Dr. R. A. Maue (Dartmouth Medical School, Hanover, NH) (26).

Primary cultures of mouse sympathetic neurons

Superior cervical ganglia were dissected from 1 day old mouse pups obtained from a breeding colony of Balb/cNctr-npc^N/+ mice established at the University of Alberta from original breeding pairs (Jackson Laboratories, Bar Harbor, ME). Mice were maintained under temperature-controlled conditions with a 12 h light/12 h dark cycle and were supplied with food and water ad libitum. Breeders were fed a 9% fat breeder diet (Purina LabDiet, Richmond, IN). Here, mice homozygous or heterozygous for the $Npc1$ mutation will be referred to as $Npc1^{-/-}$ and $Npc1^{+/-}$, respectively, whereas wild-type mice will be termed $Npc1^{+/+}$. $Npc1^{+/-}$ mice were used for breeding. Ganglia of each

mouse pup were placed separately into sterile microfuge tubes containing L15 medium supplemented with 10% rat serum and 50 ng/ml nerve growth factor. The ganglia were kept overnight at 4°C, but for no longer than 24 h, before dissociation, during which time the $Npc1$ genotype was determined from tail clippings by PCR analysis of genomic DNA using primers described previously (8). Typically, neurons were cultured for 10–12 days before the start of the experiments.

Immunocytochemistry and confocal microscopy

Neurons were fixed for 20 min at room temperature in 4% paraformaldehyde and then incubated in phosphate-buffered saline containing 10% goat serum and 50 μg/ml saponin for 2 h at room temperature. For immunocytochemical localization of synaptophysin and NPC1, the primary antibodies were mouse anti-rat synaptophysin (dilution, 1:800) and rabbit anti-mouse NPC1 (dilution, 1:500), respectively. Secondary antibodies were Texas Red-labeled goat anti-mouse IgG (dilution, 1:200) for synaptophysin and Alexa Fluor 488-labeled goat anti-rabbit IgG (dilution, 1:200) for NPC1. Images were taken using a Zeiss LSM 510 confocal laser-scanning microscope equipped with a S-Fluar 40×/1.3 oil objective (Jena, Germany). Excitation wavelengths were 488 nm (Alexa Fluor 488) and 543 nm (Texas Red).

Transfection of primary neurons with NPC1-GFP

Sympathetic neurons were grown on collagen-coated coverslips for 9 days, and then medium containing a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F10 medium containing 50 ng/ml nerve growth factor was added. This medium contained the same additives as L15 base medium except that the additional glucose and antibiotics were omitted and a B27 supplement mixture (Invitrogen) was used instead of rat serum. Adenoviruses containing a cDNA insert encoding NPC1-GFP [Ad(NPC1-GFP)] were added to the infection medium at 2 μl/ml from a stock solution of 5.77×10^{11} viral particles/ml in 10 mM Tris (pH 8.0) containing 2 mM MgCl$_2$ and 4% sucrose. Neurons were incubated in infection medium for 24 h, then in L15 base medium with rat serum for 2 days. Neurons expressing NPC1-GFP were immunostained, as described above, with anti-synaptophysin as a primary antibody.

Immunoprecipitation of NPC1 protein

The cerebellum was dissected from 5 week old $Npc1^{+/+}$ and $Npc1^{-/-}$ mice and cleaned of meninges and surface blood vessels. The remaining tissue was homogenized in buffer A [10 mM HEPES (pH 7.4) containing 150 mM NaCl, 1 mM EGTA, and 0.1 mM MgCl$_2$] with a protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Mannheim, Germany) and then centrifuged at 10,000 g for 10 min. Protein A-Sepharose beads were incubated for 16 h with anti-synaptophysin antibodies in Tris-buffered saline (pH 7.4) at 4°C, then washed twice to remove excess unbound antibodies. Control beads were incubated under similar conditions but without antibody. Beads coated with antibody were added to the supernatant resulting from a 10,000 g centrifugation of the homogenate and incubated for 2 h at room temperature. The supernatant was collected. The beads were washed four times with Tris-buffered saline and then incubated with polyacrylamide gel electrophoresis sample buffer [60 mM Tris (pH 6.8) containing 2% SDS, 150 mM β-mercaptoethanol, and 25% glycerol] for 10 min at room temperature to release bound proteins. Proteins in the bead supernatant, and proteins released from the beads, were analyzed by immunoblotting.

Isolation of synaptosomes

The cerebellum was dissected from 5 week old $Npc1^{+/+}$, $Npc1^{+/-}$, and $Npc1^{-/-}$ mice and cleaned of meninges and surface blood vessels. Synaptosomes were isolated using Percoll density centrifugation (27). Briefly, tissue was homogenized in a glass-Teflon homogenizer on ice in homogenization buffer B [10 mM HEPES (pH 7.4) containing 320 mM sucrose], diluted 2.5 times with buffer B, and centrifuged for 2 min at 3,000 g to remove nuclei and unbroken cells. The supernatant was then centrifuged at 16,000 g for 12 min. The resulting pellet was resuspended in buffer B and placed on top of a discontinuous Percoll gradient containing 4 ml each of 23, 10, and 4% Percoll in buffer B. The gradient was centrifuged in a SW40 rotor (Beckman) for 14 min at 18,300 rpm with maximum acceleration and deceleration. Synaptosomes were collected from the top of the 23% Percoll layer, diluted in Tris-buffered saline, and pelleted by centrifugation at 16,000 g for 12 min.

Subfractionation of synaptosomes

Synaptosomes were homogenized in 10 mM HEPES containing 320 mM sucrose (pH 7.4) in a glass-Teflon homogenizer, then diluted 25-fold with 5 mM HEPES-K (pH 8.0). The suspension was stirred for 30 min on ice, then centrifuged for 10 min at 8,000 g. The resulting supernatant was centrifuged at 35,000 g for 30 min, and the pellet was resuspended in 5 mM HEPES-K (pH 8.0). The suspension was placed on top of a discontinuous dextran gradient (28) consisting of 4 ml of 30% dextran, 4 ml of 18% dextran, and 3 ml of 5% dextran in Ultra-Clear centrifuge tubes (Beckman). Gradients were incubated at 4°C for 15 h before application of the sample and then centrifuged for 2 h at 23,000 rpm in a SW40 rotor (Beckman). Fractions (1 ml) were collected from the top of the gradient, diluted 10-fold with 5 mM HEPES-K (pH 7.4), and centrifuged for 3 h at 50,000 rpm in a Ti70 rotor (Beckman). The resulting pellets were resuspended in Tris-buffered saline and analyzed by immunoblotting.

Immunoblotting

Proteins were resolved by electrophoresis on 7% (for NPC1 and LAMP1) or 12% (for synaptophysin, VAMP2, and porin) polyacrylamide gels containing 0.1% SDS under reducing conditions, then transferred to polyvinylidene difluoride membranes. NPC1 was immunoblotted with rabbit polyclonal antibodies raised against a peptide of human NPC1 (dilution, 1:1,000). Immunoreactive proteins were detected by reaction with peroxidase-conjugated goat anti-rabbit IgG (dilution, 1:10,000) and visualized with ECL reagent (Amersham Biosciences, Piscataway, NJ). Antibodies used were anti-synaptophysin (dilution, 1:2,500), anti-VAMP2 (dilution, 1:5,000), and anti-porin (dilution, 1:1,000). Immunoreactive proteins were detected with peroxidase-conjugated goat anti-mouse IgG (dilution, 1:10,000) and visualized with ECL reagent. Immunoblotting for NPC2 was performed after separation of proteins by electrophoresis on 15% polyacrylamide gels containing 0.1% SDS under reducing conditions. Proteins were subsequently transferred to polyvinylidene difluoride membranes. Membranes were blocked with albumin because milk contains anti-NPC2 immunoreactive proteins. Polyclonal rabbit anti-mouse NPC2 antibodies (dilution, 1:1,000) in combination with peroxidase-conjugated goat anti-rabbit IgG (dilution, 1:10,000), and visualization with ECL reagent, were used to detect NPC2.

Isolation of synaptic vesicles

Synaptic vesicles were isolated from the cerebellum by velocity ultracentrifugation (29). Briefly, the cerebellum was dissected from 5 week old mice and cleaned of meninges and surface blood vessels. The remaining tissue was homogenized in a glass-Teflon homogenizer on ice in buffer A [10 mM HEPES (pH 7.4) with 150 mM NaCl, 1 mM EGTA, and 0.1 mM $MgCl_2$] containing a protease inhibitor cocktail (Complete Mini; Roche Diagnostics), then centrifuged at 10,000 g for 10 min. The resulting supernatant was centrifuged at 27,000 rpm in a Beckman TLA100.2 rotor for 38 min at 4°C. The pellet was collected and resuspended in buffer A and then applied to the top of a continuous glycerol gradient (5–25% glycerol in buffer A) in Ultra-Clear centrifuge tubes (Beckman). Gradients were centrifuged for 1 h at 38,000 rpm in a SW40 rotor (Beckman). Fractions were collected from the top of the tubes, and fractions that contained synaptophysin (determined by immunoblotting) were combined for lipid and protein analyses.

Lipid analyses

For determination of cholesterol content, synaptic vesicles were extracted twice with hexane-isopropanol (3:2, v/v), the organic phase was dried under a stream of nitrogen, and unesterified cholesterol was converted to its trimethylsilyl ether derivative by reaction with bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane in acetone for 15 min at 50°C. The trimethylsilyl derivative was analyzed by gas-liquid chromatography using 5α-cholestane as the internal standard. The phospholipid content of synaptic vesicles was determined as inorganic phosphorus (30). The amount of protein was determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Electron microscopy

Synaptosomes were incubated with a freshly prepared mixture of 1% paraformaldehyde and 1% glutaraldehyde in phosphate-buffered saline for 30 min on ice, then in 2% paraformaldehyde and 2% glutaraldehyde in phosphate-buffered saline for 2 h at room temperature. Synaptosomes were transferred to 0.1 M sodium cacodylate buffer (pH 7.4) and fixed in 1% osmium tetroxide in sodium cacodylate buffer for 1 h at room temperature. Fixed synaptosomes were dehydrated in solutions of increasing ethanol concentration, stained with 2% uranyl acetate in ethanol for 1 h in the dark, and embedded in resin (31). Ultrathin sections were viewed with a Hitachi H-7000 transmission electron microscope. To determine the frequency distribution of synaptic vesicle diameter, only synaptosomes with intact outer membranes were considered. Synaptic vesicle diameter was measured using the public domain software ImageJ (National Institutes of Health; http://rsb.info.nih.gov/ij/).

Statistical analyses

The statistical significance of differences ($P < 0.05$) was determined using Student's t-test.

RESULTS

NPC1 is present in distal axons of sympathetic neurons and colocalizes with synaptophysin

In neurons, late endosomes/lysosomes of the degradative pathway are generally thought to be restricted to cell bodies (24, 25). In a detailed study of the endosomal system in cultured neurons by electron microscopy after labeling with horseradish peroxidase, Parton, Simons, and Dotti (24) described early endosomes, tubulovesicular
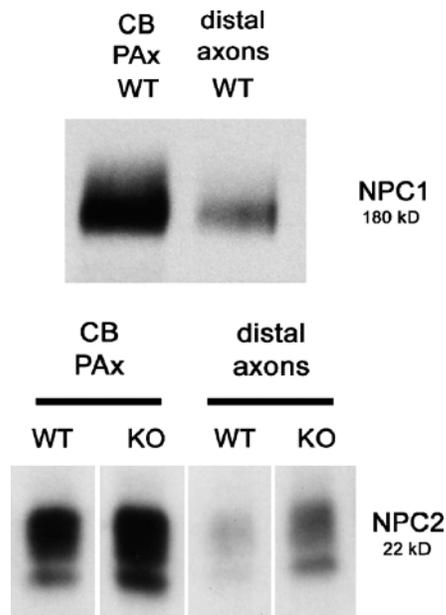
Fig. 1. Niemann-Pick type C protein 1 (NPC1) and NPC2 are present in distal axons of mouse sympathetic neurons. Cell bodies/ proximal axons (CB/PAx) and distal axons of 12 day old $Npc1^{+/+}$ [wild type (WT)] or $Npc1^{-/-}$ [knockout (KO)] sympathetic neurons cultured in compartmented dishes were harvested separately and immunoblotted for NPC1 (upper panel) and NPC2 (lower panel). An equivalent amount of protein was loaded in each lane. Data are representative of two independent experiments with similar results.

cell bodies. Similarly, electron microscopy of hippocampal slices showed that lysosomes were essentially confined to cell bodies (32). Moreover, lysosomal proteases, such as cathepsin D, are normally absent from axons (33), and in sympathetic neurons, cholesterol hydrolase activity was detected only in cell bodies (B. Karten and J. E. Vance, unpublished observations). Our previous work demonstrated that in mouse sympathetic neurons, NPC1 is present not only in neuronal cell bodies in late endosomes and lysosomes but is also abundant in vesicular structures in distal axons (23). We now confirm this observation and also show that NPC2 is present in both cell bodies/ proximal axons and distal axons of mouse sympathetic neurons (**Fig. 1**). More NPC2 protein is present in NPC1-deficient neurons than in wild-type neurons, perhaps as a compensatory response to the lack of NPC1. These observations led us to hypothesize that NPC1 plays a neuron-specific role in axons that reflects the presence of NPC1 in axonal endosomal structures that are not primarily involved in a degradative pathway.

As a first step in elucidating the mechanism of action of NPC1 in distal axons, we characterized the axonal vesicles that contained NPC1. Immunostaining of mouse sympathetic neurons with anti-NPC1 antibodies revealed that NPC1 is localized mainly to varicosities along the axons (Fig. 2A, D). Because axonal varicosities are areas of high synaptic vesicle recycling activity and neurotransmitter release (34, 35), we immunostained the axons with antibodies directed against synaptophysin, a synaptic vesicle marker protein (Fig. 2B). Synaptophysin and NPC1 significantly colocalized, particularly in the varicosities (Fig. 2C, arrows). To confirm that these observations were not attributable to nonspecific binding of anti-NPC1 antibodies, sympathetic neurons were infected with adenoviruses containing a cDNA encoding a chimera of
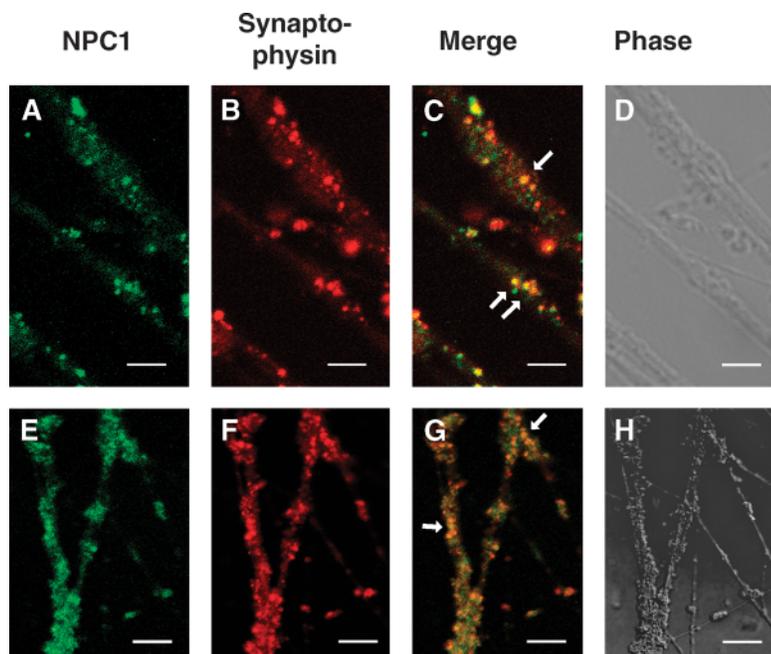
endosomal structures, and some larger spherical endosomes in axons. However, lysosomes and late endosomes, identified by horseradish peroxidase labeling after prolonged chase periods, were found nearly exclusively in the

Fig. 2. NPC1 and synaptophysin partially colocalize in axons. A–D: Twelve day old mouse sympathetic neurons were fixed with paraformaldehyde, permeabilized with saponin, incubated with antibodies raised against NPC1 (A; green) or synaptophysin (B; red), and examined by confocal microscopy. C: Overlap of staining with the two antibodies, with arrows indicating areas of colocalization. D: Phase-contrast image of the same axons. E–H: Nine day old sympathetic neurons were infected with adenovirus containing a cDNA insert encoding a chimera of NPC1 and green fluorescent protein [Ad(NPC1-GFP)]. Seventy-two hours after infection, neurons expressing NPC1-GFP (E; green) were fixed with paraformaldehyde, permeabilized with saponin, and incubated with antibodies raised against synaptophysin (F; red). G: Overlap of GFP fluorescence and staining for synaptophysin. Arrows indicate areas of colocalization. H: Phase-contrast image of the same axons. Data are representative of two independent experiments with similar results. Bars = 5 µm.

NPC1 fused to GFP (Fig. 2E). This NPC1-GFP construct has previously been characterized and drives the expression of a functional NPC1 protein that eliminates the late endosomal accumulation of cholesterol in mouse $Npc1^{-/-}$ sympathetic neurons (26). When neurons expressing NPC1-GFP were stained with anti-synaptophysin antibodies (Fig. 2F), the NPC1 chimera was found to colocalize, at least partially, with synaptophysin (Fig. 2G, arrows) in a pattern similar to that seen with anti-NPC1 antibodies (Fig. 2C, arrows). Thus, NPC1 and synaptophysin significantly, but not completely, colocalize in axons.

We next investigated whether or not NPC1 and synaptophysin reside on the same vesicles. A homogenate of mouse cerebellum was immunoprecipitated, in the absence of detergent to preserve vesicle integrity, using beads coated with anti-synaptophysin antibodies (**Fig. 3A**). The immunoprecipitate was immunoblotted with anti-NPC1 antibodies. As shown in Fig. 3A, NPC1 was present in the anti-synaptophysin immunoprecipitate. In accor-
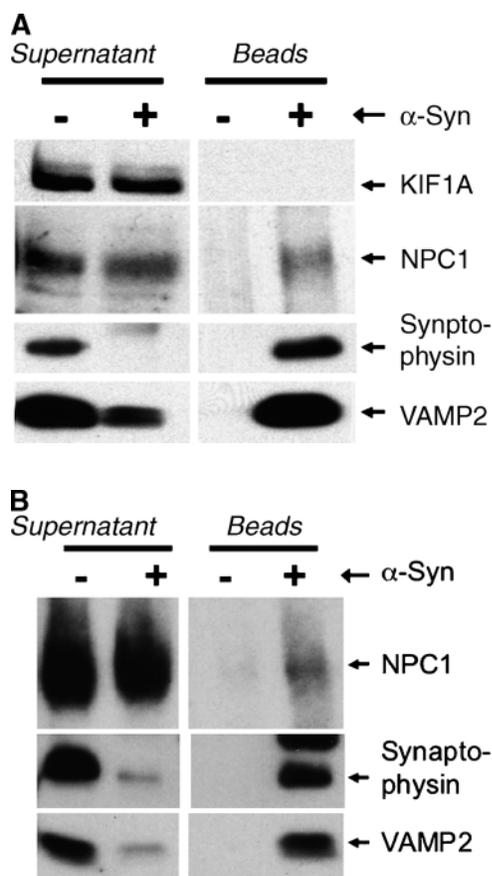
dance with the immunofluorescence microscopy studies, showing that NPC1 and synaptophysin partially colocalize (Fig. 2), not all NPC1 was immunoprecipitated with anti-synaptophysin antibodies (Fig. 3A). As expected, VAMP2, a synaptic vesicle protein and a known binding partner of synaptophysin, bound to the beads coated with anti-synaptophysin. In contrast, the cytosolic motor protein kinesin family member 1a (negative control) remained in the supernatant from the immunoprecipitation. Beads not coated with antibody were used as a control for nonspecific binding. A parallel immunoprecipitation procedure was performed in cell lysates from sympathetic neurons and yielded similar results (i.e., NPC1 was immunoprecipitated with anti-synaptophysin antibodies) (Fig. 3B). These data indicate that a neuronal vesicle population exists that contains both NPC1 and synaptophysin.

NPC1 is present in endosomal structures in nerve endings

To determine whether NPC1 was present in central nervous system synapses, we prepared synaptosomes, which represent isolated nerve endings, from the cerebellum of wild-type and $Npc1^{-/-}$ mice. Synaptosomal proteins were immunoblotted for NPC1. The NPC1 protein was clearly present in cerebellar synaptosomes from $Npc1^{+/+}$ but not $Npc1^{-/-}$ mice (**Fig. 4A**).

The presynaptic nerve terminal contains not only synaptic vesicles but also mitochondria and endosomal structures that participate in the synaptic vesicle recycling pathway. Therefore, we isolated synaptic vesicles from synaptosomes by glycerol gradient velocity centrifugation (29) and determined the localization of NPC1. NPC1 was not present in the purified synaptic vesicles (Fig. 4B). Interestingly, however, NPC1 and the late endosomal marker protein LAMP1 cofractionated in the bottom fraction of the gradient (Fig. 4B, fraction 1). To test whether or not NPC1 was present in endosomes in the nerve ending, we ruptured synaptosomes from $Npc1^{+/+}$ mice by hypoosmotic shock to release intrasynaptosomal vesicles and organelles. Upon centrifugation of the resulting mixture at 38,000 g, synaptic vesicles remained in the supernatant. Organelles in the pellet were then separated by centrifugation over a dextran gradient. Fractions from the gradient were immunoblotted for NPC1 and for proteins characteristic of synaptic vesicles (synaptophysin, VAMP2), endosomes (LAMP1), and mitochondria (porin). NPC1 cofractionated with the endosomal marker protein LAMP1 as well as with VAMP2 and the remaining synaptophysin (fractions 4 and 5 in Fig. 4C). The mitochondrial marker protein, porin, was found mainly in fractions of higher density (fractions 9 and 10), although some immunoreactive porin was also found contaminating the endosomal fraction (Fig. 4C). These observations demonstrate that in the presynaptic nerve ending, NPC1 is present in recycling endosomes but is absent from purified synaptic vesicles.

Fig. 3. A vesicle population in the cerebellum contains both NPC1 and synaptophysin. Protein A-Sepharose beads were incubated with or without anti-synaptophysin (α-Syn) antibodies, then added to a 10,000 g supernatant of a homogenate of mouse cerebellum (A) or mouse sympathetic neurons (B) for 2 h at room temperature. Proteins bound to the beads and in the supernatant were analyzed by immunoblotting for kinesin family member 1a KIF1A (negative control), NPC1, synaptophysin, and vesicle-associated membrane protein-2 (VAMP2). Data are representative of two independent experiments with similar results.

Cholesterol content of synaptic vesicles from $Npc1^{-/-}$ cerebellum is normal, whereas protein content is altered

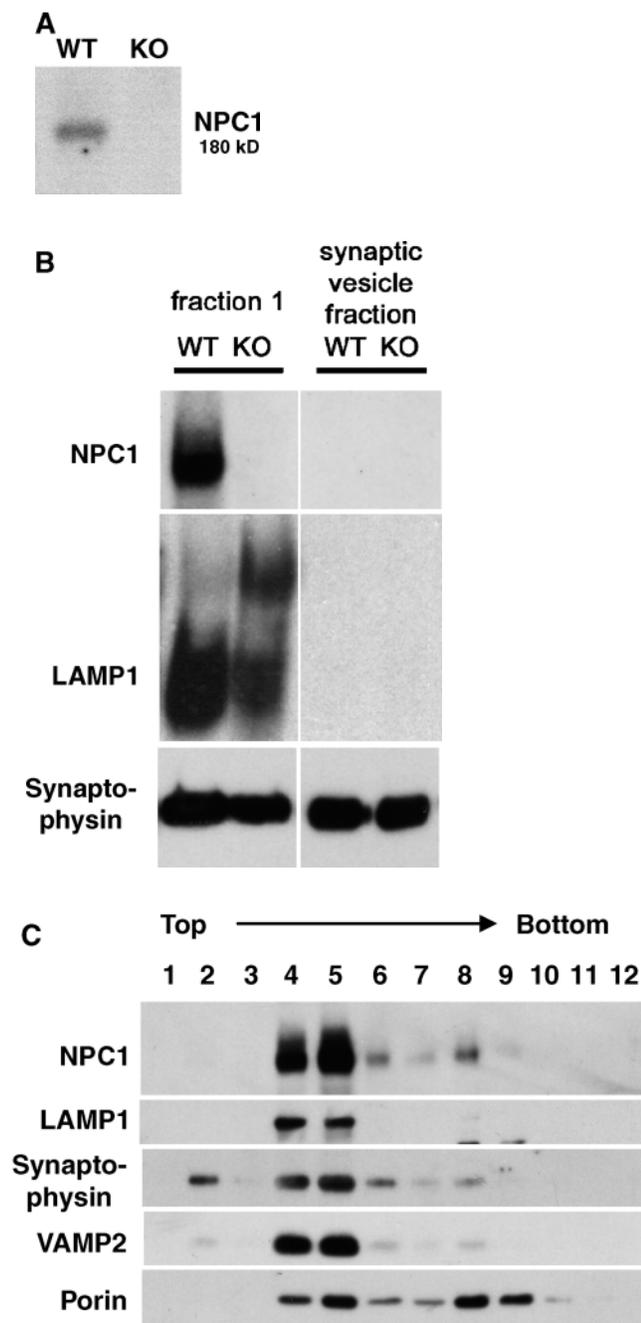One likely explanation for the presence of NPC1 in endosomal structures in the presynaptic nerve terminal is

Fig. 4. NPC1 is present in recycling endosomes in the presynaptic terminal. A: Synaptosomes were isolated from the cerebellum of 5 week old *Npc1*$^{+/+}$ [wild type (WT)] and *Npc1*$^{-/-}$ [knockout (KO)] mice and analyzed by immunoblotting for the presence of NPC1. B: The 28,000 *g* supernatants from homogenates of the cerebellum of 5 week old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice were separated over a linear glycerol gradient by velocity ultracentrifugation. The synaptic vesicle fraction and the bottom fraction of the gradient (fraction 1) were collected and immunoblotted for NPC1, lysosome-associated membrane protein-1 (LAMP1), and synaptophysin. C: Synaptosomes from wild-type mice were lysed, and then intrasynaptosomal membranes (excluding synaptic vesicles) were pelleted at 38,000 *g* and separated by ultracentrifugation over a dextran gradient. Fractions were collected from the top of the gradient and immunoblotted for NPC1, LAMP1, synaptophysin, VAMP2, and porin. Data are representative of two independent experiments with similar results.

that NPC1 participates in the endosomal synaptic vesicle recycling pathway. If NPC1 played a role in this pathway, one might expect that NPC1 deficiency would alter the composition of synaptic vesicles. Therefore, we determined whether the lack of NPC1 altered the cholesterol content of synaptic vesicles from mouse cerebellum. During the isolation of synaptic vesicles by glycerol gradient ultracentrifugation, it became apparent that synaptic vesicles isolated from the NPC1-deficient cerebellum had a lower sedimentation coefficient than did vesicles from wild-type cerebellum (**Fig. 5A**). This observation indicated that synaptic vesicles from *Npc1*$^{-/-}$ mice had either a lower specific density or an increased size compared with synaptic vesicles from *Npc1*$^{+/+}$ cerebella. Neither the cholesterol-to-protein ratio (Fig. 5B) nor the cholesterol-to-phospholipid ratio (Fig. 5C) was altered by NPC1 deficiency. In contrast, immunoblotting for two synaptic vesicle proteins, synaptophysin and VAMP2, revealed that the amount of synaptophysin relative to VAMP2 in synaptic vesicles from *Npc1*$^{-/-}$ mice was higher than that in synaptic vesicles from *Npc1*$^{+/+}$ mice (Fig. 5D). Thus, the protein composition of synaptic vesicles isolated from mouse cerebellum is altered by NPC1 deficiency.

NPC1 deficiency causes biochemical and morphological changes in the presynaptic terminal

Because the composition of synaptic vesicles was modified by NPC1 deficiency, we next determined whether or not the protein composition of the presynaptic terminal as a whole was altered. Synaptosomes were isolated from the cerebellum of 5 week old *Npc1*$^{+/+}$, *Npc1*$^{+/-}$, and *Npc1*$^{-/-}$ mice and analyzed by immunoblotting for proteins characteristic of organelles in the nerve ending (**Fig. 6A**). Growth-associated protein-43, which is present in the plasma membrane of nerve endings (36) but is not actively involved in synaptic vesicle recycling, was used as a loading control. The amounts of the synaptic vesicle proteins synaptophysin and VAMP2 were unaltered by NPC1 deficiency. Thus, the increased synaptophysin/VAMP2 ratio in pure synaptic vesicles from *Npc1*$^{-/-}$ mice (Fig. 5D) is a consequence of an altered distribution of these proteins rather than of a change in overall concentration of one of these proteins in the entire presynaptic terminal. Interestingly, compared with synaptosomes from *Npc1*$^{+/-}$ and *Npc1*$^{+/+}$ mice, synaptosomes from *Npc1*$^{-/-}$ cerebellum are enriched in the late endosomal/lysosomal protein LAMP1 (Fig. 6A). Moreover, in synaptosomes, the late endosomal protein NPC2 is also increased markedly by NPC1 deficiency (Fig. 6A), consistent with the finding that NPC2 (another endosomal/lysosomal protein) in distal axons is increased by NPC1 deficiency (Fig. 1).

Examination of *Npc1*$^{+/+}$ synaptosomes by electron microscopy revealed uniformly sized synaptic vesicles (Fig. 6B). In contrast, *Npc1*$^{-/-}$ synaptosomes contained a population of aberrantly large vesicular structures that were not observed in wild-type synaptosomes (Fig. 6B). Although the frequency distribution of the diameters of synaptic vesicles was similar in *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$
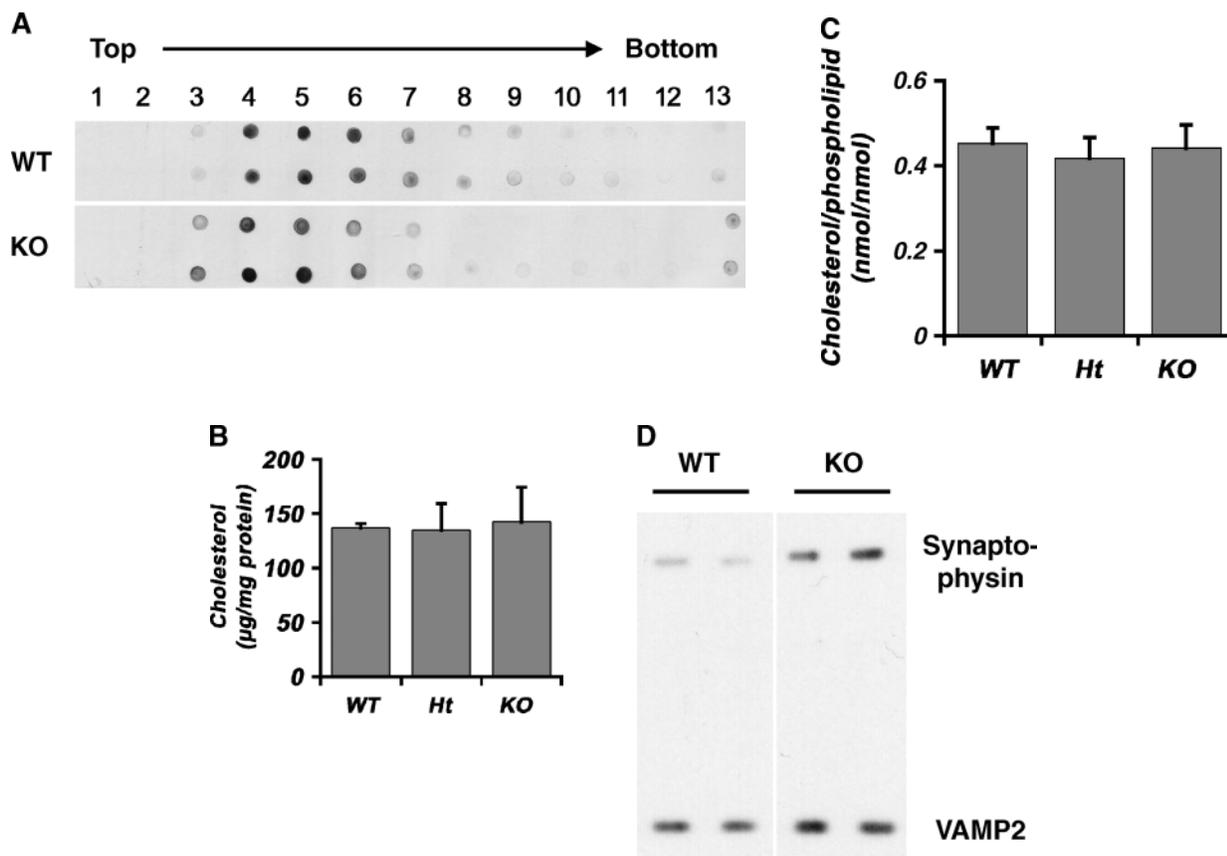
Fig. 5. Characteristics of synaptic vesicles from $Npc1^{+/+}$ and $Npc1^{-/-}$ cerebellum. A: Pure synaptic vesicles were isolated from the cerebellum of 5 week old $Npc1^{+/+}$ [wild type (WT)] and $Npc1^{-/-}$ [knockout (KO)] mice by velocity ultracentrifugation over a linear glycerol gradient. Fractions were collected and analyzed by dot blotting for synaptophysin immunoreactivity. B, C: Fractions 3–7 (synaptic vesicles) were combined and analyzed for cholesterol (B) and phospholipid (C) content. Data shown are means ± SD from four independent synaptic vesicle preparations. D: Synaptic vesicles from $Npc1^{+/+}$ or $Npc1^{-/-}$ cerebellum were separated by denaturing gel electrophoresis, with equal amounts of cholesterol loaded per lane, and analyzed by immunoblotting for synaptophysin and VAMP2. Shown are representative replicates of two independent experiments with similar results, each with duplicate preparations. Ht, heterozygote.

synaptosomes, with the majority having a diameter of ~40 nm (Fig. 6C), $Npc1^{-/-}$ synaptosomes contained a significant number of vesicles with diameters of >75 nm (Fig. 6C, inset).

These data demonstrate that NPC1 is present in recycling endosomes of the presynaptic terminal. The absence of NPC1 leads to morphological changes in the presynaptic terminal and alterations in the protein composition of synaptic vesicles.

DISCUSSION

Despite a growing interest in NPC disease, the exact functions of NPC1 and NPC2 remain elusive. Key questions underlying the molecular basis of NPC disease include the following: Why does the loss of function of a ubiquitously expressed protein affect mainly the central nervous system? And why are neurons particularly vulnerable to the loss of function of NPC1? One feasible explanation for the severe neurological problems in individuals with NPC disease is that although NPC1 is expressed in all

cell types examined to date, NPC1 might have an additional function in neurons. Another possibility is that peripheral cells, but not neurons, operate a pathway that partially compensates for NPC1 deficiency. Alternatively, the function of NPC1 might be the same in all cell types, but neurons might simply be particularly sensitive to the loss of NPC1 function.

Subcellular localization of NPC1 and NPC2

To address the first possibility, that NPC1 plays a neuron-specific role in addition to its role in all cells, we focused on our observation that NPC1 is present in distal axons. We show that *i*) NPC1 and NPC2 are present in distal axons of mouse sympathetic neurons; *ii*) the NPC1 protein is located in varicosities in distal axons and extensively colocalizes with the synaptic vesicle marker synaptophysin; *iii*) a population of endosomal vesicles in synaptosomes contains both NPC1 and synaptophysin, and these vesicles are likely to be recycling endosomes in the presynaptic nerve terminal; *iv*) synaptic vesicles from $Npc1^{-/-}$ mice have normal amounts of cholesterol and
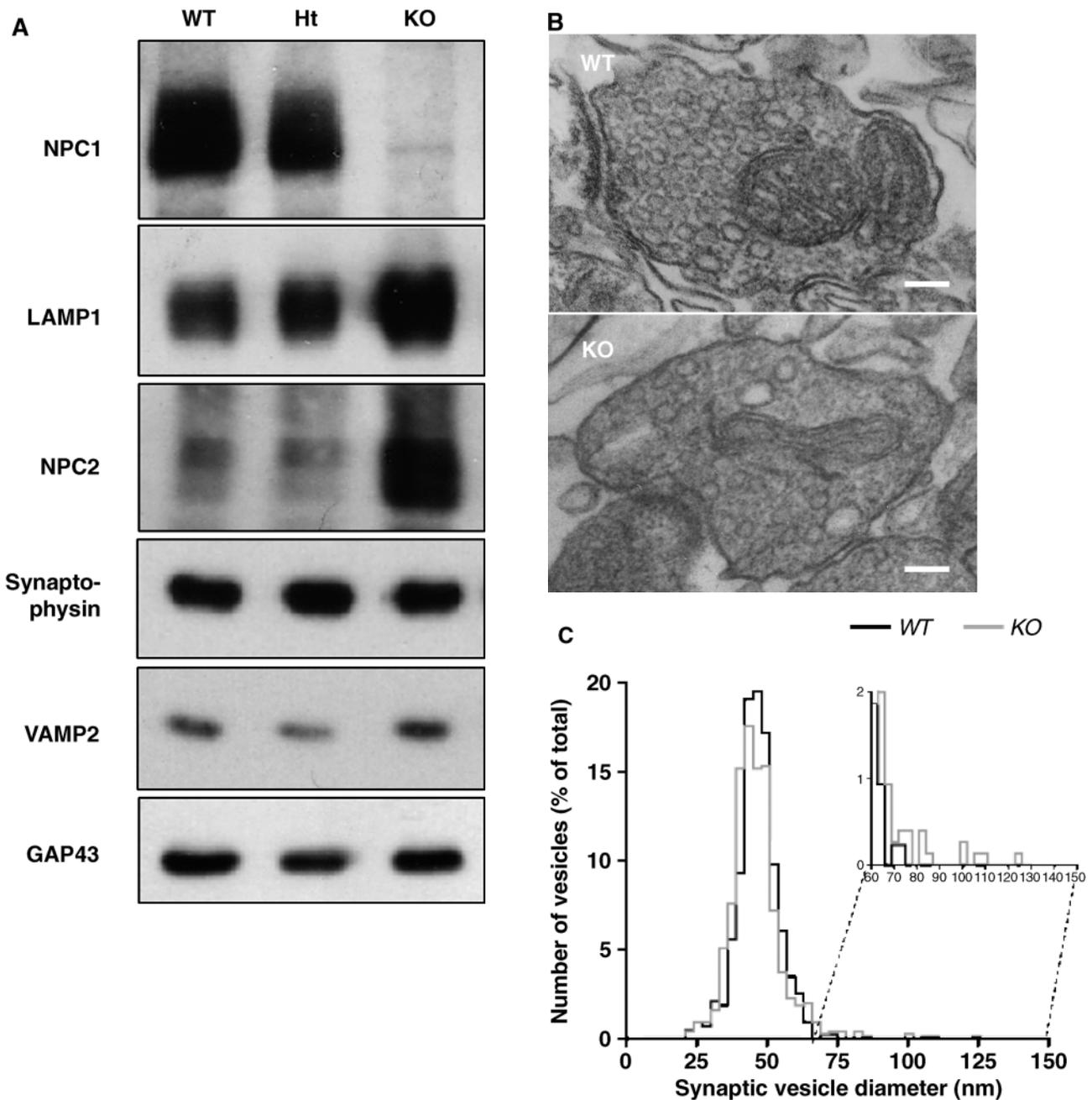
Fig. 6. Biochemical and morphological changes in NPC1-deficient presynaptic terminals. A: Synaptosomes were isolated from the cerebellum of 5 week old $Npc1^{+/+}$ [wild type (WT)], $Npc1^{+/-}$ [heterozygote (Ht)], and $Npc1^{-/-}$ [knockout (KO)] mice and immunoblotted for NPC1, LAMP1, NPC2, synaptophysin, and VAMP2. Growth-associated protein-43 (GAP43) was used as a loading control. B: Synaptosomes from $Npc1^{+/+}$ and $Npc1^{-/-}$ mice were fixed in paraformaldehyde and glutaraldehyde, stained with osmium tetroxide and uranyl acetate, and embedded in resin. Ultrathin sections were examined by electron microscopy. Bars = 100 nm. Images are representative of three independent experiments. C: Electron microscopic images were analyzed using ImageJ software to determine synaptic vesicle diameter. Only vesicles in synaptosomes with an intact outer membrane were taken into consideration. The percentage of vesicles of each diameter was plotted against diameter. The total number of vesicles measured was 518 for $Npc1^{+/+}$ synaptosomes and 812 for $Npc1^{-/-}$ synaptosomes from three independent preparations.

phospholipid but exhibit differences in sedimentation behavior and protein composition compared with synaptic vesicles from $Npc1^{+/+}$ mice; and v) NPC1-deficient synaptosomes contain a population of aberrantly large vesicles, indicating that NPC1 is necessary to ensure the correct morphology of the presynaptic nerve ending.

In the classic model of NPC1 function, NPC1 is involved in exporting cholesterol and/or other lipids from late endosomes and lysosomes to the endoplasmic reticulum and plasma membrane. Our previous observations are consistent with this model in that cholesterol accumulates within late endosomes/lysosomes in cell bodies of $Npc1^{-/-}$

neurons but is relatively depleted in axons (22) and the movement of cholesterol from cell bodies to distal axons is impaired (23). In neurons, degradative late endosomes and lysosomes are localized mainly to cell bodies (24, 25). We found, however, that NPC1 and NPC2 are present not only in cell bodies but also in distal axons (23) (Fig. 1). We hypothesized, therefore, that NPC1 might play a role in distal axons beyond the distribution of lipids out of late endosomes/lysosomes.

According to our immunocytochemical studies, NPC1 extensively colocalizes with synaptophysin and is most abundant in varicosities and branch points along the axons. In addition, NPC1 is found in synaptosomes isolated from mouse cerebellum. However, NPC1 is not a component of synaptic vesicles per se but, rather, is a component of endosomes in the nerve terminal. These observations raise the possibility that NPC1 is involved in synaptic vesicle recycling. Interestingly, ceroid lipofuscinosis neuronal 3, another endosomal/lysosomal protein, whose absence causes the neurological lysosomal storage disease juvenile neuronal ceroid lipofuscinosis (Batten disease) (reviewed in 37), but whose function is as yet unknown, has likewise recently been detected in recycling endosomes of the presynaptic terminal (38).

Possible role of NPC1 in presynaptic terminals

Neurotransmitter release from the presynaptic terminal involves a series of events termed "synaptic vesicle re-
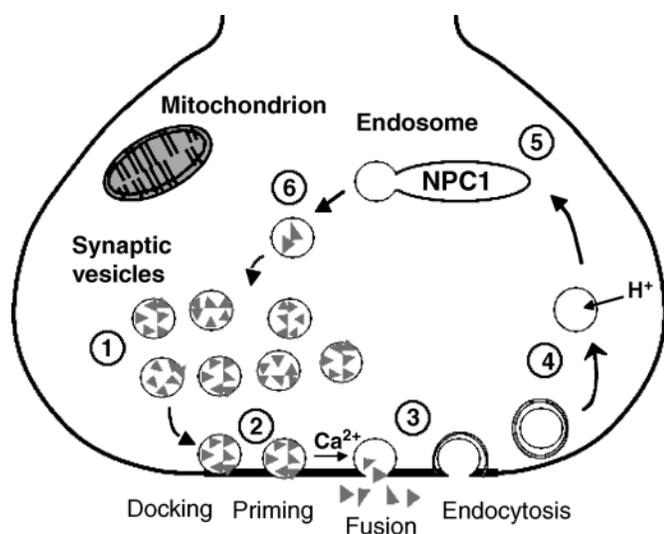

Fig. 7. Proposed function of NPC1 in the synaptic vesicle recycling pathway in a nerve ending (synaptosome). (1) A pool of synaptic vesicles filled with neurotransmitter (triangles) is present in the vicinity of the presynaptic plasma membrane. (2) Synaptic vesicles dock with the active zone and are primed for fusion. (3) Neurotransmitters are released into the synaptic cleft after a fusion pore opens between vesicles and the plasma membrane. (4) Synaptic vesicles are reinternalized by clathrin-mediated endocytosis. (5) After shedding the clathrin coat and reacidification, vesicles reach an endosomal compartment containing NPC1. (6) Synaptic vesicles are reformed from the recycling endosome and refilled with neurotransmitter.

cycling" (reviewed in 39), elements of which are depicted in **Fig. 7**, which shows a nerve terminal, or a synaptosome, with synaptic vesicles. In this process, synaptic vesicles are filled with neurotransmitter by active transport. The amount of neurotransmitter is regulated by the size of the synaptic vesicle. The vesicle fuses with the plasma membrane, and after pore opening and the concomitant release of neurotransmitter, synaptic vesicles are refilled with neurotransmitter by three alternative pathways. First, vesicles can be reacidified and refilled with neurotransmitter while remaining docked. Second, vesicles can undock but remain close to the active zone, where they are acidified and refilled with neurotransmitter. Third, as illustrated in Fig. 7, vesicles can be endocytosed via clathrin-coated pits and then refilled with neurotransmitter either after being uncoated or after passing through an endosomal intermediate. Our experiments indicate that NPC1 is present in this endosomal intermediate.

The endosomal recycling pathway is slower than the other mechanisms that do not involve clathrin-mediated endocytosis, but it is thought to have higher capacity than the other pathways and to be crucial for maintaining a continuous release of neurotransmitter (39). If NPC1 were involved in the endosomal recycling pathway, elimination of NPC1 might lead to a slower replenishment of the releasable pool of synaptic vesicles. Such a defect would not completely block neurotransmitter release but might account for some of the progressive neurological problems encountered over the lifetime of an individual with NPC disease. Reduced electrical activity and changes in the intrinsic excitability of $Npc1^{-/-}$ Purkinje cells have been reported (40). Endosomal recycling might also provide a quality control mechanism for the removal of defective synaptic vesicles from the recycling pool upon passing through the endosome (39). If NPC1 played a role in this quality control process, changes in synaptic vesicle composition or structure would be expected in the absence of NPC1. Indeed, we found two prominent aberrations in nerve endings from NPC1-deficient cerebellum: altered protein composition of synaptic vesicles and the presence of enlarged vesicles in the nerve terminal.

The increased ratio of synaptophysin to VAMP2 that we observed in synaptic vesicles from $Npc1^{-/-}$ mice provides an interesting link to cholesterol metabolism. Synaptophysin is a cholesterol binding protein (41) that appears to be localized to detergent-resistant membrane domains (42) and forms a complex with VAMP2. This complex mediates the tethering of synaptic vesicles to the presynaptic plasma membrane (39). Formation of a synaptophysin/VAMP2 complex depends on the cholesterol content of the membrane and was shown to be reduced in the brains of $Npc1^{-/-}$, compared with $Npc1^{+/+}$, mice (42). We speculate that NPC1 controls local cholesterol concentrations in the nerve terminal, thereby regulating the sorting of synaptophysin into synaptic vesicles and its interaction with VAMP2.

The origin of the larger vesicles that we observed by electron microscopy of NPC1-deficient cerebellar synaptosomes is intriguing. These vesicles are likely to be

enlarged synaptic vesicles that have escaped the quality control mechanism that normally involves NPC1. However, because in synaptosomes the endosomal proteins LAMP1 and NPC2 are increased by NPC1 deficiency, we cannot exclude the possibility that these enlarged vesicles represent an increased number, or increased size, of recycling endosomes in the presynaptic terminal (43). Such a proliferation of endosomes might represent a compensatory mechanism for overcoming NPC1 deficiency in the synapse. Alternatively, these vesicles might be enlarged synaptic vesicles that have arisen from uncontrolled homotypic fusion events resulting from subtle changes in membrane structure imparted by recycling through an NPC1-deficient endosome. The fusion of synaptic vesicles has been linked to Rab5 function (44), and interestingly, a deficient recycling of GM1 ganglioside from Rab5-containing endosomes to the plasma membrane has been observed in NPC1-deficient fibroblasts (45).

In summary, we report that NPC1 is present in endosomes in the presynaptic terminal of neurons. The absence of NPC1 causes morphological changes in the presynaptic nerve ending as well as alterations in the composition of synaptic vesicles. These findings suggest a neuron-specific role for NPC1 in the synaptic vesicle recycling pathway, in addition to its established role in cholesterol export from late endosomes/lysosomes. These alterations, therefore, might contribute to the severe neurological phenotype characteristic of NPC1 disease.▮

REFERENCES

1. Ikonen, E., and M. Holtta-Vuori. 2004. Cellular pathology of Niemann-Pick type C disease. *Semin. Cell Dev. Biol.* **15:** 445–454.
2. Chang, T. Y., P. C. Reid, S. Sugii, N. Ohgami, J. C. Cruz, and C. C. Chang. 2005. Niemann-Pick type C disease and intracellular cholesterol trafficking. *J. Biol. Chem.* **280:** 20917–20920.
3. Liscum, L. 2000. Niemann-Pick type C mutations cause lipid traffic jam. *Traffic.* **1:** 218–225.
4. Pentchev, P. G., M. T. Vanier, K. Suzuki, and M. C. Patterson. 1995. Niemann-Pick disease type C: a cellular cholesterol lipidosis. *In* The Metabolic and Molecular Bases of Inherited Disease. A. L. Beaudet, C. R. Scriver, W. S. Sly, and D. Valle, editors. McGraw Hill, New York. 2625–2639.
5. Sturley, S. L., M. C. Patterson, W. Balch, and L. Liscum. 2004. The pathophysiology and mechanisms of NP-C disease. *Biochim. Biophys. Acta.* **1685:** 83–87.
6. Neufeld, E. B., M. Wastney, S. Patel, S. Suresh, A. M. Cooney, N. K. Dwyer, C. F. Roff, K. Ohno, J. A. Morris, E. D. Carstea, et al. 1999. The Niemann-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. *J. Biol. Chem.* **274:** 9627–9635.
7. Higgins, M. E., J. P. Davies, F. W. Chen, and Y. A. Ioannou. 1999. Niemann-Pick C1 is a late endosome-resident protein that transiently associates with lysosomes and the trans-Golgi network. *Mol. Genet. Metab.* **68:** 1–13.
8. Loftus, S. K., J. A. Morris, E. D. Carstea, J. Z. Gu, C. Cummings, A. Brown, J. Ellison, K. Ohno, M. A. Rosenfeld, D. A. Tagle, et al. 1997. Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. *Science.* **277:** 232–235.
9. Carstea, E. D., J. A. Morris, K. G. Coleman, S. K. Loftus, D. Zhang, C. Cummings, J. Gu, M. A. Rosenfeld, W. J. Pavan, D. B. Krizman, et al. 1997. Niemann-Pick C1 disease gene: homology to mediators of cholesterol momeostasis. *Science.* **277:** 228–231.
10. Sokol, J., E. J. Blanchette-Mackie, H. S. Kruth, N. K. Dwyer, L. M. Amende, J. D. Butler, E. Robinson, S. Patel, R. O. Brady, M. E. Comly, et al. 1988. Type C Niemann-Pick disease. *J. Biol. Chem.* **263:** 3411–3417.
11. Liscum, L., R. M. Ruggiero, and J. R. Faust. 1989. The intracellular transport of low density-derived cholesterol is defective in Niemann-Pick type C fibroblasts. *J. Cell Biol.* **108:** 1625–1636.
12. Naureckiene, S., D. E. Sleat, H. Lackland, A. Fensom, M. T. Vanier, R. Wattiaux, M. Jadot, and P. Lobel. 2000. Identification of HE1 as the second gene of Niemann-Pick C disease. *Science.* **290:** 2298–2301.
13. Friedland, N., H. L. Liou, P. Lobel, and A. M. Stock. 2003. Structure of a cholesterol-binding protein deficient in Niemann-Pick type C2 disease. *Proc. Natl. Acad. Sci. USA.* **100:** 2512–2517.
14. Ko, D. C., J. Binkley, A. Sidow, and M. P. Scott. 2003. The integrity of a cholesterol-binding pocket in Niemann-Pick C2 protein is necessary to control lysosome cholesterol levels. *Proc. Natl. Acad. Sci. USA.* **100:** 2518–2525.
15. Liscum, L., and S. L. Sturley. 2004. Intracellular trafficking of Niemann-Pick C proteins 1 and 2: obligate components of subcellular lipid transport. *Biochim. Biophys. Acta.* **1685:** 22–27.
16. Cruz, J. C., S. Sugii, C. Yu, and T-Y. Chang. 2000. Role of Niemann-Pick type C1 protein in intracellular trafficking of low density lipoprotein-derived cholesterol. *J. Biol. Chem.* **275:** 4013–4021.
17. Cruz, J. C., and T-Y. Chang. 2000. Fate of endogenously synthesized cholesterol in Niemann Pick type C1 cells. *J. Biol. Chem.* **275:** 41309–41316.
18. Goldin, E., C. F. Roff, S. P. F. Miller, C. Rodriguez-Lafrasse, M. T. Vanier, R. O. Brady, and P. G. Pentchev. 1992. Type C Niemann-Pick disease: a murine model of the lysosomal cholesterol lipidosis accumulates sphingosine and sphinganine in liver. *Biochim. Biophys. Acta.* **1127:** 303–311.
19. Zervas, M., K. L. Somers, M. A. Thrall, and S. U. Walkley. 2001. Critical role for glycosphingolipids in Niemann-Pick disease type C. *Curr. Biol.* **11:** 1283–1287.
20. Watanabe, Y., S. Akaboshi, G. Ishida, T. Takeshima, T. Yano, M. Taniguchi, K. Ohno, and K. Nakashima. 1998. Increased levels of GM2 ganglioside in fibroblasts from a patient with juvenile Niemann-Pick disease type C. *Brain Dev.* **20:** 95–97.
21. Karten, B., H. Hayashi, G. A. Francis, R. B. Campenot, D. E. Vance, and J. E. Vance. 2005. Generation and function of astroglial lipoproteins from Niemann-Pick type C1-deficient mice. *Biochem. J.* **387:** 779–788.
22. Karten, B., D. E. Vance, R. B. Campenot, and J. E. Vance. 2002. Cholesterol accumulates in cell bodies, but is decreased in distal axons, of Niemann-Pick C1-deficient neurons. *J. Neurochem.* **83:** 1154–1163.
23. Karten, B., D. E. Vance, R. B. Campenot, and J. E. Vance. 2003. Trafficking of cholesterol from cell bodies to distal axons in Niemann Pick C1-deficient neurons. *J. Biol. Chem.* **278:** 4168–4175.
24. Parton, R. G., K. Simons, and C. G. Dotti. 1992. Axonal and dendritic endocytic pathways in cultured neurons. *J. Cell Biol.* **119:** 123–137.
25. Parton, R. G., and C. G. Dotti. 1993. Cell biology of neuronal endocytosis. *J. Neurosci. Res.* **36:** 1–9.
26. Paul, C. A., P. C. Reid, A. K. Boegle, B. Karten, M. Zhang, Z. G. Jiang, D. Franz, L. Lin, T. Y. Chang, J. E. Vance, et al. 2005. Adenovirus expressing an NPC1-GFP fusion gene corrects neuronal and nonneuronal defects associated with Niemann Pick type C disease. *J. Neurosci. Res.* **81:** 706–719.
27. Dunkley, P. R., J. W. Heath, S. M. Harrison, P. E. Jarvie, P. J. Glenfield, and J. A. Rostas. 1988. A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: homogeneity and morphology of subcellular fractions. *Brain Res.* **441:** 59–71.

28. Goncalves, P. P., and A. P. Carvalho. 1997. Membrane potential manipulation in synaptic plasma membrane vesicles for studying neurotransmitter uptake and release. *Brain Res. Brain Res. Protoc.* **1:** 1–12.

29. Clift-O'Grady, L., A. D. Linstedt, A. W. Lowe, E. Grote, and R. B. Kelly. 1990. Biogenesis of synaptic vesicle-like structures in a pheochromocytoma cell line PC-12. *J. Cell Biol.* **110:** 1693–1703.

30. Zhou, X., and G. Arthur. 1992. Improved procedures for the determination of lipid phosphorus by malachite green. *J. Lipid Res.* **33:** 1233–1236.

31. Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26:** 31–43.

32. Bednarski, E., C. E. Ribak, and G. Lynch. 1997. Suppression of cathepsins B and L causes a proliferation of lysosomes and the formation of meganeurites in hippocampus. *J. Neurosci.* **17:** 4006–4021.

33. Sahenk, Z., J. N. Whitaker, and J. R. Mendell. 1990. Immunocytochemical evidence for the retrograde transport of intraaxonal cathepsin D: possible relevance to the dying-back process. *Brain Res.* **510:** 1–6.

34. Buckley, K. M., and S. C. Landis. 1983. Morphological studies of synapses and varicosities in dissociated cell cultures of sympathetic neurons. *J. Neurocytol.* **12:** 67–92.

35. Buckley, K. M., and S. C. Landis. 1983. Morphological studies of neurotransmitter release and membrane recycling in sympathetic nerve terminals in culture. *J. Neurocytol.* **12:** 93–116.

36. Van Lookeren Campagne, M., A. B. Oestreicher, P. M. Van Bergen en Henegouwen, and W. H. Gispen. 1990. Ultrastructural double localization of B-50/GAP43 and synaptophysin (p38) in the neonatal and adult rat hippocampus. *J. Neurocytol.* **19:** 948–961.

37. Phillips, S. N., J. W. Benedict, J. M. Weimer, and D. A. Pearce. 2005. CLN3, the protein associated with Batten disease: structure, function and localization. *J. Neurosci. Res.* **79:** 573–583.

38. Luiro, K., O. Kopra, M. Lehtovirta, and A. Jalanko. 2001. CLN3 protein is targeted to neuronal synapses but excluded from synaptic vesicles: new clues to Batten disease. *Hum. Mol. Genet.* **10:** 2123–2131.

39. Sudhof, T. C. 2004. The synaptic vesicle cycle. *Annu. Rev. Neurosci.* **27:** 509–547.

40. Paul, C. A., A. K. Boegle, and R. A. Maue. 2004. Before the loss: neuronal dysfunction in Niemann-Pick type C disease. *Biochim. Biophys. Acta.* **1685:** 63–76.

41. Thiele, C., M. J. Hannah, F. Fahrenholz, and W. B. Huttner. 2000. Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. *Nat. Cell Biol.* **2:** 42–49.

42. Mitter, D., C. Reisinger, B. Hinz, S. Hollmann, S. V. Yelamanchili, S. Treiber-Held, T. G. Ohm, A. Herrmann, and G. Ahnert-Hilger. 2003. The synaptophysin/synaptobrevin interaction critically depends on the cholesterol content. *J. Neurochem.* **84:** 35–42.

43. Marxen, M., W. Volknandt, and H. Zimmermann. 1999. Endocytic vacuoles formed following a short pulse of K$^+$-stimulation contain a plethora of presynaptic membrane proteins. *Neuroscience.* **94:** 985–996.

44. Shimizu, H., S. Kawamura, and K. Ozaki. 2003. An essential role of Rab5 in uniformity of synaptic vesicle size. *J. Cell Sci.* **116:** 3583–3590.

45. Sugimoto, Y., H. Ninomiya, Y. Ohsaki, K. Higaki, J. P. Davies, Y. A. Ioannou, and K. Ohno. 2001. Accumulation of cholera toxin and GM1 ganglioside in the early endosome of Niemann-Pick C1-deficient cells. *Proc. Natl. Acad. Sci. USA.* **98:** 12391–12396.