Rapid and Gentle Immunopurification of Brain Synaptic Vesicles

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Current methods to isolate synaptic vesicles (SVs), the organellar quanta of synaptic transmission, require highly specialized materials and up to 24 h. These technical obstacles have thus far limited the study of SVs in models of synaptic function and pathophysiology. Here, we describe techniques for the rapid isolation of SVs by immunoprecipitation with widely available antibodies conjugated to magnetic beads. We report that the inexpensive rho1D4 monoclonal antibody binds SVs and show that elution with the 1D4 peptide yields native vesicles that are ≥10-fold purer than those obtained with classical techniques. These methods substantially widen the accessibility of SVs, enabling their purification in 60–90 min for downstream analyses including mass spectrometry and cryo-electron microscopy. Immunopurified SV preparations from mouse brain contained apolipoprotein E, the LDL receptor Lrp1, and enzymes involved in lipid metabolism, suggesting that SVs may play direct roles in lipid homeostasis and lipoprotein trafficking at the nerve terminal.

Key words: biochemistry; cryo-electron microscopy; lipid metabolism; neurodegeneration; proteomics; synaptic vesicles

Significance Statement

SVs are small organelles that form and recycle at nerve terminals to enable synaptic transmission. Much remains unknown about the processes that enable the formation and function of SVs. Moreover, nerve terminals appear to be particularly vulnerable to pathophysiologic processes underlying neurodegenerative diseases and schizophrenia. Although techniques to purify synaptic vesicles thus have the potential to yield significant insights into physiology and pathophysiology of nerve terminals, current methods rely on either esoteric materials or expression of transgenes. This article addresses these problems by establishing robust, efficient methods for SV purification using widely available materials, and it highlights several promising areas of future study arising from proteomic analyses of immunopurified SVs.

Introduction

Synaptic vesicles (SVs) are small (~40 nm diameter) organelles that store neurotransmitters within nerve terminals and rapidly undergo exocytosis upon presynaptic Ca2+ influx. The current standard for high-yield, high-purity SV purification for biochemical analysis relies on the size, physical properties, and abundance of these organelles in the brain (Ahmed et al., 2013). Based on techniques developed in the 1960s (Whittaker et al., 1964), this approach takes ~24 h and involves gradient centrifugation and sedimentation of vesicles at high speed before size exclusion chromatography. The chromatography media typically used for the final size-exclusion step, controlled pore glass or Sephacryl S-1000, are no longer widely available, and these preparations are unavoidably contaminated with other cellular components such as glutamine synthetase (Ahmed et al., 2013; Taoufiq et al., 2020). Other approaches involving purification of SVs by immunoprecipitation (IP) have been limited by elution steps that damage or destroy the resin-bound vesicles (Burger et al., 1989; Takamori et al., 2000; Chantranupong et al., 2020). These techniques have been valuable in determining SV membrane composition (Takamori et al., 2006), neurotransmitter content (Burger et al., 1989, 1991; Chantranupong et al., 2020), and extending areas of future study arising from proteomic analyses of immunopurified SVs.

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and transport activity (Burger et al., 1989, 1991; Takamori et al., 2000). However, each of these approaches also requires highly specialized materials, such as large quantities of proprietary antibody or the expression of exogenous genes, and may yield vesicles of reduced purity compared with classical methods (Chantharupong et al., 2020). Investigators seeking deeper insights into the biogenesis, composition, physical properties, function, and pathophysiological relevance of SVs would be substantially aided by gentler, more selective, and more convenient procedures for their isolation.

This article describes advances to immunoprecipitation-based approaches for SV purification that substantially reduce the barrier to entry for obtaining pure SVs. We demonstrate the suitability of these approaches for analysis of SVs by cryo-electron tomography and liquid chromatography-mass spectrometry (LC-MS). Our LC-MS proteomics results provide unequivocal evidence of the purity of these preparations and suggest previously unappreciated roles for SVs in lipid metabolism and lipoprotein trafficking.

**Materials and Methods**

**Animals.** C57B/6J mice of either sex between 14 and 20 d of age were used for all experiments. All work was conducted according to protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee.

**Antibodies for immunoprecipitation.** Mouse monoclonal anti-syt1 (synaptotagmin-1) antibody (mAb 48; Matthew et al., 1981) and anti-SV2 (Buckley and Kelly, 1985) antibodies were purified by protein G chromatography from stocks of ascites generated before the year 2010. Rho1D4 mAb was purchased from the University of British Columbia (https://ubc.flintbox.com). Indistinguishable results were obtained using two batches of rho1D4 mAb manufactured in 2017 and 2020. Bovine IgG was purchased from Sigma-Aldrich. For most experiments, all antibodies were dialyzed extensively against PBS (140 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4) before bead coupling, but indistinguishable results were also obtained using rho1D4 directly as provided by the manufacturer in PBS. All antibodies were aliquoted and stored frozen at −20°C before bead coupling. We observed loss of activity in rho1D4 antibody aliquots that underwent evaporation during storage at −20°C and thus recommend that care be taken to ensure this does not occur, for example, by storing at lower temperatures.

**Bead preparation.** Dynabeads M-270 epoxy (300 mg, catalog #14302D, Thermo Fisher Scientific) were stored at 30 mg/ml in anhydrous, amine-free N,N-dimethylformamide (DMF) for up to 18 months before coupling. Nonautoclaved tubes were used for all steps involving Dynabeads. For each coupling reaction, 10 mg beads were transferred to a fresh 1.7 ml polypropylene microcentrifuge tube, and the DMF was removed after collecting the slurry. Beads were then washed (2 × 1 ml washes total, alternat- ing strokes in a Teflon-glass Dounce homogenizer with rotation at 850–900 rpm using a digital overhead mixer (IEKA). The homogenate was then centrifuged (20 min, 35,000 × g, 1°C). During centrifugation, 3 mg of Ab-Dynabeads (−100 µl slurry) were transferred to a fresh 2 ml microcentrifuge tube, washed with 1 × 1 ml wash buffer (150 mM KCl, 10 mM potassium phosphate buffer, pH 7.2, at 0°C), and resuspended with 100 µl homogenization buffer. Following centrifugation, the supernatants (−2.5 mg/ml protein) were pooled, and 1.9 ml was added to each tube containing Ab-Dynabeads. The tubes were incubated with rotation for 25 min, with the temperature maintained at 0°C by placing the tubes inside 50 ml conical tubes packed with ice. The beads were then collected using a magnetic stand, the supernatant was discarded, and the beads were washed four times by gently resuspending and triturating in 1 ml ice-cold wash buffer. In all cases, the final wash was used to transfer the beads to fresh 1.7 ml microcentrifuge tubes for elution. For Figure 1, the beads were split into two equal portions (1.5 mg each). For protein analysis, one portion was eluted by adding 30 µl 2% SDS and 25 mM Tris, pH 8.0, and heating to 50°C for 5 min. For polar amine analysis, the other portion was eluted by adding 30 µl of 50:50 MeOH:borate buffer and incubating on ice for 5 min. In Figure 1, immunoprecipitations using all four Ab-Dynabead conjugates were conducted in parallel for each experiment. For native elution experiments, the rho1D4 beads (3 mg; see Fig. 3) bearing SVs were washed as above and incubated first with 50 µl 200 µM 1D4 peptide (Cube Biotech) for 30 min on ice. This eluate was transferred to a fresh tube, and the beads were then eluted in 50 µl 2% SDS with heating to 50°C for 5 min. The elution buffers included 100 mM sodium borate, pH 8.5 (see Fig. 3) or 140 mM NaCl and 25 mM HEPES-NaOH, pH 7.4 (see Fig. 4). SVs were readily eluted with the 1D4 peptide in all buffers tested, for example, containing the following (in mM): 135 NaCl; 25 HEPES-NaOH, pH 7.4; 5 EDTA; 100 KCl; 25 HEPES-NaOH, pH 7.4; and 200 ammonium acetate (see Fig. 4). In later experiments (data not shown), higher concentrations of peptide (−1 mg/ml) appeared more effective for eluting SVs, and so we encourage investigators using this method to titrate this peptide elution step during initial studies.

**SDS-PAGE and immunoblot.** Following elution with peptide or SDS, one-third volume of 4× SDS sample buffer containing β-mercaptoethanol or dithiothreitol (DTT) was added to the eluate, and the input fraction was prepared by mixing 30 µl brain supernatant with 60 µl 4× SDS sample buffer. Samples were reduced and denatured by heating to 50°C for 15 min and subjected to SDS-PAGE on 4–20% polyacrylamide gradient gels (TGX, Bio-Rad). For protein analysis, 15 µl of each sample was loaded, and the gel was fixed in MeOH-acetic acid and stained with Lumitine One-Step fluorescent protein stain (Biotium; Fig. 1, see Fig. 3). For immunoblots as shown in Figure 1, the same amount of input was run, but the immunoprecipitation samples were diluted 10-fold in sample buffer before SDS-PAGE. Proteins were transferred to PVDF membranes, blocked in 5% nonfat dry milk in 150 mM NaCl, 10 mM Tris, pH 7.4, plus 0.1% Tween 20 (TBS-T), and incubated overnight with primary antibody in TBS-T containing 1% nonfat dry milk. The primary antibodies were used in the following: mouse anti-synaptobrevin monoclonal (clone 69.1, 1:1000 dilution of an 0.1 mg/ml stock; catalog #104 211, Synaptic Systems), guinea pig anti-synaptophysin polyconal (1:1000 dilution of a 0.5 mg/ml stock; catalog #101 004, Synaptic Systems), mouse anti-synaptotagmin monoclonal (mAb 48, 1:1000 dilution of a 3.7 mg/ml stock purified from ascites fluid; Developmental Studies Hybridoma Bank), mouse anti-syt1 monoclonal (clone HPC-1, 1:1000 dilution of a 3 mg/ml stock; catalog #ab63265, Abcam), and rabbit polyclonal anti-VDAC (voltage-dependent anion channel; 1:1000 SV immunoisolation. All buffers and equipment were cooled to 0–1°C before beginning experiments, and all operations from homogenization until bead elution were conducted in a cold room. One to two C57Bl/6J mice, postnatal day (P) 14–20, were killed, and the brains, including cerebellum and brainstem, were rapidly removed. Each brain was homogenized in 4.2 ml of homogenization buffer [125 mM KCl, 20 mM potassium phosphate buffer, 5 mM EGTA, and protease inhibitors (Complete Mini EDTA-free, 1 tablet/10 ml), pH 7.3, at 0°C], using 10 strokes in a Teflon-glass Dounce homogenizer with rotation at 850–900 rpm using a digital overhead mixer (IEKA). The homogenate was then centrifuged (20 min, 35,000 × g, 1°C). During centrifugation, 3 mg of Ab-Dynabeads (−100 µl slurry) were transferred to a fresh 2 ml microcentrifuge tube, washed with 1 × 1 ml wash buffer (150 mM KCl, 10 mM potassium phosphate buffer, pH 7.2, at 0°C), and resuspended with 100 µl homogenization buffer. Following centrifugation, the supernatants (−2.5 mg/ml protein) were pooled, and 1.9 ml was added to each tube containing Ab-Dynabeads. The tubes were incubated with rotation for 25 min, with the temperature maintained at 0°C by placing the tubes inside 50 ml conical tubes packed with ice. The beads were then collected using a magnetic stand, the supernatant was discarded, and the beads were washed four times by gently resuspending and triturating in 1 ml ice-cold wash buffer. In all cases, the final wash was used to transfer the beads to fresh 1.7 ml microcentrifuge tubes for elution. For Figure 1, the beads were split into two equal portions (1.5 mg each). For protein analysis, one portion was eluted by adding 30 µl 2% SDS and 25 mM Tris, pH 8.0, and heating to 50°C for 5 min. For polar amine analysis, the other portion was eluted by adding 30 µl of 50:50 MeOH:borate buffer and incubating on ice for 5 min. In Figure 1, immunoprecipitations using all four Ab-Dynabead conjugates were conducted in parallel for each experiment. For native elution experiments, the rho1D4 beads (3 mg; see Fig. 3) bearing SVs were washed as above and incubated first with 50 µl 200 µM 1D4 peptide (Cube Biotech) for 30 min on ice. This eluate was transferred to a fresh tube, and the beads were then eluted in 50 µl 2% SDS with heating to 50°C for 5 min. The elution buffers included 100 mM sodium borate, pH 8.5 (see Fig. 3) or 140 mM NaCl and 25 mM HEPES-NaOH, pH 7.4 (see Fig. 4). SVs were readily eluted with the 1D4 peptide in all buffers tested, for example, containing the following (in mM): 135 NaCl; 25 HEPES-NaOH, pH 7.4; 5 EDTA; 100 KCl; 25 HEPES-NaOH, pH 7.4; and 200 ammonium acetate (see Fig. 4). In later experiments (data not shown), higher concentrations of peptide (−1 mg/ml) appeared more effective for eluting SVs, and so we encourage investigators using this method to titrate this peptide elution step during initial studies.
Figure 1. Immunopurification of synaptic vesicles. A, Scheme for vesicle immunoprecipitation and analysis. Each mouse brain provided sufficient material for analysis of protein and neurotransmitter using two different mAbs. B, Staining of proteins separated by SDS-PAGE demonstrates broad similarity among anti-SV2, anti-synt1, and rho1D4 immunoprecipitates, with minimal protein binding by control beads bearing pooled bovine IgG. Note the dominant band at 38 kDa, corresponding to synaptophysin. C, Immunoblot analysis of precipitated material. Each antibody yields strong enrichment of SV proteins, but only the expected weak enrichment of the plasma membrane t-SNARE syntaxin-1 and no detectable contamination from the mitochondrial protein VDAC. D, For each experiment, the area of the HPLC fluorescence peak corresponding to NBD-derivatized glutamate (B) or GABA (E) was plotted against the normalized intensity of the synaptophysin band on immunoblot (n = 4 biological replicates using two separately prepared batches of Ab-Dynabeads for each mAb; B–E). Example raw chromatograms used for GABA and glutamate measurements are shown in Extended Data Figure 1-1. "LU"s, luminescence units = seconds.

**Amine analysis.** For experiments in Figure 1, 8 µl of bead eluate in 50/50 MeOH:borate buffer was combined with 4 µl of freshly prepared derivatization solution comprising 5 mg/ml NBD-Cl (4-chloro-7-nitro-1,2,3-benzoxadiazole) in MeOH and heated to 60°C for 70 min on a PCR block. 7-nitro-1,2,3-benzoxadiazole (NBD) derivatized amines were analyzed on an HPLC system (Agilent Infinity 1260 Bio-Inert) with a reversed-phase C18 column (Agilent Peptide Mapping, 2.1 x 150 mm, 2.7 µm particle size) and fluorescence detector (Agilent 1260 FLD Spectra, 470 nm excitation, 530 nm emission). The instrument was operated using a manual injector, the column was kept at 40°C, and samples were applied to overfill a 5 µl sample loop. Mobile phase A was 95:5 H2O:MeCN containing 10 mM ammonium acetate, and mobile phase B was 95:5 MeCN:H2O containing 5 mM ammonium acetate. For Figure 1, GABA and glutamate were separated using an optimized gradient as follows: 0–20 min, 10–100% B; 20–25 min, 100% B; 25–30 min, 100–10% B. Peak identities were confirmed by running authentic standards, and areas were determined by integration in Agilent Chemstation software.

Dynamic light scattering and negative-stain transmission electron microscopy. Three microliters of rho1D4 peptide eluate were analyzed in a micro cuvette on a dynamic light-scattering instrument (Wyatt). For each sample, 10 acquisitions were performed, and data from those acquisitions showing good autocorrelation functions (>90% of scans) were averaged to produce a single replicate. Data corresponding to the distribution of particle diameters were exported and plotted in Prism. Transmission electron microscopy (TEM) samples were prepared by uranyl formate staining of peptide-eluted SVs on glow-discharged grids.

Cryo-EM sample preparation and analysis. Rho1D4 beads (3 mg) bearing SVs from one-half mouse brain were eluted in 50 µl of HEPES elution buffer containing the following (in µM): 25 HEPES, 140 NaCl, and 200 1D4 peptide. For cryoEM, 3 µl of the purified SV suspension was adsorbed onto 2 nm carbon-layer-coated Quantifoil R1.2/1.3300 mesh copper grids, glow discharged for 2 s at 4°C and 95% humidity using a Vitrobot Mark IV (Thermo Fisher Scientific). Ten nanometer gold fiducial particles were used for tilt-series alignment. All images were acquired on a Talos Arctica 200 kV TEM using a Gatan K3 direct electron detector in correlated double-sampling counting mode, with an energy filter set to a 20 eV slit width. Nineteen tilt series were recorded at 31,000 X magnification (2.89 Å/pixel), from −48 to 48° with 3° increments, at a dose rate of 15e−/Å²/s and defocus of −3 µm. Tilt series alignment and tomographic reconstruction were done using the IMOD package. After preprocessing to remove x-ray artifacts and coarse alignment, a seed model was generated using 25 seed points. A fiducial model was generated by tracking individual seed points and filling the gaps. The fiducial model was then edited by moving the residuals to the center of the gold particles, if not already there. Sample tomogram thickness was set to 500 arbitrary units, and the tomogram was binned down by three to improve contrast. A boundary model was created with six contour lines enclosing the sample at three different Y locations in the tomogram to ensure the specimen is flat and centered along the Z axis. The final alignment stack was then generated, and the tomogram was
For each SV, diameters along the longest axis and the axis perpendicular to it were measured manually in IMOD (outer layer to outer layer of the lipid bilayer). To determine the longest axis, multiple axes at the largest cross section of the SV were measured, and the longest among them was chosen. The ratio of the diameters was used to calculate ellipticity. Small beads (100 nm) were plotted using Prism, and 3D rendering of the SVs was done using IMOD.

**Sample preparation for LC-MS.** SVs from 0.5–1 mouse brain, eluted by treatment with SDS (2% w/v) or 1D4 peptide (200 μM), were prepared for liquid chromatography and mass spectrometry (LC-MS) using the SP3 method (Hughes et al., 2019). Ten percent w/v SDS was added to peptide-eluted samples to reach a final SDS concentration of 2% w/v. Reduction and denaturation was achieved by adding DTT (100 mM freshly prepared aqueous stock solution) to a final concentration of 5 mM and incubating at 50°C for 25 min. Cysteine alkylation was achieved by adding iodoacetamide (200 mM freshly prepared aqueous stock solution) to a final concentration of 15 mM and incubating at room temperature in the dark for 30 min, followed by quenching of iodoacetamide by further addition of DTT (26 mM DTT total). Dynabeads M-270 carboxyllic acid (catalog #14305D, Thermo Fisher Scientific) were then added (2 μg/μl final concentration), and the tubes were mixed well, followed by the addition of 1 volume of absolute ethanol and brief incubation on a thermomixer (5 min, 1000 rpm, 23°C) to drive protein adsorption to the beads. The beads were washed three times with 200 μl 80% ethanol and transferred to a fresh tube with the final wash. Following removal of the supernatant of the SVs, Graphites were eluted from the beads by overnight digestion in 25–30 μl trypsin solution (0.01 μg/μl in 100 mM ammonium bicarbonate; catalog #V5111, Promega) with shaking in a thermomixer (1000 rpm, 37°C). Eluates from this step were used directly for LC-MS. The data presented in Figure 2 and Extended Data Figure 2-1 were obtained from experiments independent of those presented in Figure 5 and Extended Data Figure 5-1.

**LC-MS data acquisition.** For Figure 2 and Extended Data Figure 2-1, data were acquired using a quadrupole time-of-flight mass spectrometer (Impact II, Bruker) connected to a nanoflow HPLC system (nanoACQUITY, Waters) via an electrospray ionization source. The HPLC was equipped with a nano-HPLC column [Waters ACQUITY UPLC Peptide BEH C18, 1.7 μm particles, 20 cm long, 75 μm inner diameter (i.d.)], preceded by a trap column (Waters ACQUITY UPLC M-Class Symmetry C18, 5 μm particles, 20 mm long, 180 μm i.d.), and held at 60°C. Mobile phase A was 0.2% formic acid in H2O, and mobile phase B was 0.2% formic acid in 80:20 MeCN:H2O. Injection volume was 3 μl. MS1 survey scan. Precursor ions were dynamically excluded for 20 s with a 20 ppm mass tolerance after being selected for fragmentation. LC-MS data analysis: Raw files were directly analyzed using the FragPipe data processing pipeline (Kong et al., 2017). Peptide spectral matching was performed using a mouse proteome database downloaded from Uniprot in June 2021 with the following settings: precursor mass tolerance, 50 ppm; fragment mass tolerance, 20 ppm; mass calibration and parameter optimization enabled; up to 2 missed cleavages; peptide length, 7–50; peptide mass range, 500–5000 Da. Cysteine carboximide-thiolation was selected as a fixed modification and methionine oxidation and N-terminal acetylation as variable modifications. PeptideProphet and ProteinProphet were used for validation and assignment of peptide and protein results. Samples from each experiment were analyzed together using IonQuant (Yu et al., 2021) with matched-between-runs (MBR) enabled and label-free quantification with normalization between experiments performed using the MaxLFQ algorithm with the following settings: feature detection m/z tolerance 10 ppm, feature detection retention time tolerance 0.4 min, MBR retention time (RT) tolerance, 1 min; MBR min correlation, 0; MBR ion false discovery rate (FDR), 0.01; MBR top runs 10; MBR peptide FDR 1; MBR protein FDR 1. Razor intensities for selected proteins were plotted in Prism (GraphPad). For comparison with data from Taucuflg et al. (2020), proteins detected in at least one experiment were included, and gene names were homologized using the SynGO ID convert tool ([https://www.synogonportal.org/convert.html](https://www.synogonportal.org/convert.html); Koopmans et al., 2019) before analysis of overlap using R.

**Data availability.** The raw data (see Fig. 5) are freely available online via the Chorus project at [https://chorusproject.org/anonymous/download/experiment-/8025126159725249575](https://chorusproject.org/anonymous/download/experiment-/8025126159725249575).
The putative identities of major contaminants in the rho1D4 immunoprecipitates, which included four bands; 90–150 and 250 kDa, were established by LC-MS (see below). Fluorescence HPLC analysis of polar amines eluted from the beads demonstrated the predominance of glutamate and GABA, as expected for pure SVs isolated at 0°C (Fig. 1D–E; Extended Data Fig. 1-1; Burger et al., 1991; Chantranupong et al., 2020). These data likewise indicate the absence of intact lysosomes or mitochondria, which contain many other polar amines (Chen et al., 2016; Abu-Remaileh et al., 2017). Beads coated with pooled bovine IgG did not immunoprecipitate SV proteins (Fig. 1) or vesicular amino acid neurotransmitters (Extended Data Fig. 1-1). The amount of neurotransmitter immunoprecipitated by each antibody correlated well with the yield of synaptophysin, further suggesting that these antibodies pull down similar sets of vesicles despite differences in affinity for SVs (SV2 > synt1 > rho1D4; Fig. 1 D–E).

Proteomic analysis of synt1– and rho1D4 immunoprecipitates using trypsin digestion and LC-MS (Fig. 2, Extended Data Fig. 2-1) confirmed the purity of the immunoprecipitated material. In α-syt1 immunoprecipitates, well-known SV proteins made up nearly all the most abundant proteins detected (Fig. 2A,
Extended Data Data Fig. 2-1). These proteins include those observed by Western blot (Fig. 1), vesicular transporters for GABA and glutamate, and a wide assortment of Rab GTPases and other proteins associated with SVs (Takamori et al., 2006; Taoufiq et al., 2020; Fig. 2A). Subunits of both the V₀ and V₁ sectors of the vesicular ATPase were among the most intense observed proteins, also in accordance with previous studies (Taoufiq et al., 2020). As expected from the protein bands observed by SDS-PAGE (Fig. 1), SV proteins were observed in similar intensity in the rho1D4 bead-bound material (Fig. 2A), again demonstrating that the rho1D4 mAb immunoprecipitates a population of vesicles similar to that obtained with antibodies against SV proteins. The primary presumed contaminants in the rho1D4 bead-bound material included the Rho guanine nucleotide exchange factor Arhgef2 (predicted mass ∼112 kDa), dipetidyl peptidase 8 (Dpp8, 102 kDa), protein Furry homolog (Fry, 339 kDa), nicotinamide riboside kinase 1 (22.3 kDa), and zinc finger MYM-type protein 2 (Znym2, 155 kDa), along with a number of ribosomal proteins (Fig. 2B). These observations correspond well to the protein banding pattern observed in SDS-PAGE for rho1D4 immunoprecipitates (Fig. 1).

Although the anti-syt1 and anti-SV2 mAbs are highly effective for SV immunoprecipitation (Fig. 1), they do not readily allow for elution of native vesicles for downstream applications. The 9-mer 1D4 peptide sequence (TETSQVAPA) has been commercialized as a C-terminal affinity tag, as this peptide gently elutes rho1D4 mAb–bound targets under native conditions (MacKenzie et al., 1984; Wong et al., 2009). We thus attempted elution of rho1D4 bead-bound SVs with the 1D4 peptide (Fig. 3A) and found that a single incubation with the 1D4 peptide released ∼25% of the bead-bound protein, including ∼50% of the bead-bound synaptophysin (Fig. 3B). This eluate, which typically contained ∼25 ng/µl protein (Fig. 3B, Table 1), represents an enrichment of vesicular protein by several hundred-fold (Table 1). Examination of this eluate by dynamic light scattering demonstrated a population of particles 30–60 nm in diameter (Fig. 3C). Negative-stain TEM of these samples likewise demonstrated a population of vesicular structures 30–60 nm in diameter, studded with ∼8 nm tall structures that may represent the V₁ sector of the V-ATPase (Fig. 3D).

We characterized peptide-eluted SV samples, which to our knowledge represent the most intact preparations of free SVs reported to date, with cryo-electron tomography (Fig. 4). We observed a striking variety of SV morphologies in our preparation, including oblong or angulated shapes, in addition to the expected spheres. Measurement of the longest and orthogonal diameters of each vesicle (Fig. 4B,C) yielded an average longest diameter of 43.4 nm, an average orthogonal diameter of 39.1 nm, and an average ellipticity of 1.11 (Fig. 4C). To our knowledge, this is the first characterization of nonspherical morphology in isolated SVs, as previous cryo-EM studies of purified SVs assumed a spherical shape in their morphologic characterization (Takamori et al., 2006). However, we note that studies of SVs in situ have identified nonspherical SVs for decades (Korneliussen, 1972; Rastad, 1981; Tao et al., 2018), although the causes of nonspherical SV morphology remain unknown. Even with the relatively lower contrast provided by cryo-EM without negative staining, electron densities on the outer surface of the SV membranes were observed (Fig. 4A).

We next performed proteomics experiments with deeper coverage enabled by high resolution and accurate mass measurements using an Orbitrap mass spectrometer and optimized
Table 1. Purity and yield of SVs isolated by rho1D4-IP

| Fraction                        | Volume, µl | [Protein], ng/µl | [Synaptophysin], normalized | Enrichment factor | Percentage of input synaptophysin | Preparation time, minutes |
|---------------------------------|------------|------------------|-----------------------------|-------------------|-----------------------------------|---------------------------|
| Input (supernatant from one-half mouse brain) | 1900       | 2500 ± 300       | 1                           | 1                 | 100                               | 30                        |
| rho1D4 bead-bound material      | ~60        | 95 ± 11          | 7.7 ± 0.7                   | 180 ± 30          | ~20%                              | 60                        |
| 1D4 peptide eluate             | ~60        | 25 ± 7.5         | 3.3 ± 0.6                   | 330 ± 120         | ~10%                              | 90                        |

Quantification of input protein was achieved by BCA assay, and quantification of SV yields was achieved as described in Materials and Methods. Data are shown as mean ± SEM for three immunoprecipitations using two mouse brains and three batches of rho1D4 beads. In contrast, SVs prepared using classical methods usually achieve synaptophysin enrichment factors of 20–30 when adjusted for the type of input fraction used here (Ahmed et al., 2013), whereas the SV-tag approach achieves an enrichment factor of 10–15 for synaptobrevin (Chantranupong et al., 2020).

Figure 4. Cryo-electron tomography of synaptic vesicles obtained by rho1D4-IP. A, Varied morphology of SVs as seen by cryo-electron microscopy and their corresponding three-dimensional reconstructions. Scale bar, 25 nm. B, Frequency distribution of SV diameters measured along the longest axis (n = 421). C, Frequency distribution of SV ellipticity defined as the ratio between the longest and orthogonal diameters of each vesicle.

A comparison of material obtained by syt1-IP and rho1D4-IP with peptide elution demonstrated a robust correlation between intensity scores of proteins detected in each sample (Fig. 5B), indicating that each IP method isolates a similar population of SVs. Even with peptide elution, Rho1D4-IP vesicles did contain some residual contamination, including a small cluster of ribosomal subunits (Rps, Rpl proteins) and initiation factor subunits (eIF proteins) enriched in these samples (Fig. 5B). Abundant contaminants in the classical SV preparation of Taoufiq et al. (2020), including glutamine synthetase and GAPDH, were observed in low abundance or were undetectable in our preparations (Extended Data Figs. 2-1, 5-1, 5-2). Among proteins observed in our IP samples but not in classical preparations, RNA-processing proteins predominated, suggesting that both rho1D4 and α-syt1 IP procedures may isolate small amounts of RNA and RNA-associated proteins (Extended Data Fig. 5-2). We note that ribosomal subunits have also been detected in classical SV preparations (Taoufiq et al., 2020) as well as in axonal proteomes (Chuang et al., 2018; Hobson et al., 2022), although the connection between ribosomal proteins and axonal membrane trafficking is not well defined.

We focused our annotation of these data on proteins that may modulate the lipid composition of nerve terminals because much remains unknown about the processes underlying lipid metabolism and homeostasis at nerve terminals. Most notably, we detected the lipoprotein apolipoprotein E (ApoE), along with several other apolipoproteins and the lipoprotein receptor Lrp1, in all SV preparations analyzed (Fig. 5C; Extended Data Figs. 2-1, 5-1). ApoE was among the top 80 most abundant proteins by MaxLFQ intensity in syt1 and rho1D4 peptide-eluted immunoprecipitates, and both ApoE and Lrp1 were previously detected in studies of classically isolated SVs (Takamori et al., 2006; Taoufiq et al., 2020). Several other proteins involved in lipid transport and metabolism were among the 500 most abundant proteins by MaxLFQ intensity (Fig. 5C), including the phospholipid-transporting ATPases Atp8a1 and Atp9a, fatty acyl-CoA synthetase ACSBG1, monoglyceride lipase, and the enzyme choline-phosphate cytidylyltransferase A (Pcyt1a), which plays a key role in regulating phosphatidylcholine (PC) abundance. These proteins, with the exception of Pcyt1a, were likewise detected...
immunomodulatory protein (Tjoelker et al., 1995). Together, these findings indicate that SVs contain not only a host of enzymes involved in regulating membrane composition but also ApoE and Lrp1, which allow for ApoE internalization and recycling. These results, which are consistent with studies demonstrating the accumulation of ApoE and Lrp1 at nerve terminals (Bilousova et al., 2019), implicate the SV cycle in ApoE-mediated transport processes and suggest previously unappreciated roles for the SV cycle in the pathophysiology of neuroinflammation (Guttenplan et al., 2021) and age-related neurodegeneration (Bilousova et al., 2019).

Discussion

This work details improved approaches for SV immunopurification (Matthew et al., 1981; Burger et al., 1989) and introduces rho1D4-IP as an accessible method for the purification and elution of SVs. Rho1D4-IP with peptide elution enables the rapid (<2 h) isolation of an exceptionally pure SV sample (Fig. 3, Table 1) without contamination from antibodies, exposure to proteases, or harsh physicochemical conditions. Future experiments may determine whether these vesicles undergo their native biochemical functions including membrane fusion and neurotransmitter loading. If native vesicles (Figs. 3-5) are not needed, techniques involving harsh elution from immunobeads provide a favorable compromise of purity for yield, as this material is still at least fivefold more enriched for SV proteins than SVs prepared classically (~20–30-fold enrichment, Ahmed et al., 2013) or by using the SV-tag approach (~10–15-fold enrichment, Chanthanupong et al., 2020; Table 1). Although it does not enable molecular specification of target vesicle populations like SV-tag, rho1D4-IP represents a promising example of antibody repurposing and enables wide access to a new standard in purity and convenience for purifying SVs. The molecular interactions underlying the avidity of rho1D4 mAb-coated beads for SVs remain unclear but likely involve low-affinity binding between rho1D4 mAb and SV proteins along with polyvalency effects from multiple binding sites on both the bead surface and on each SV.

We emphasize the suitability of these approaches for studies with transgenic mice; a single P10–P20 mouse brain (0.3–0.4 g) comfortably provides enough material for two IP experiments (3 mg Ab-Dynabeads each) including protein and neurotransmitter analyses (Fig. 1). mAbs against syt1 and SV2 also provide a highly pure SV sample (Figs. 1, 2, 5, Extended Data Figs. 2-1, 5-1), although they do not presently allow for native vesicle

using classical SV isolation methods (Taoufiq et al., 2020). Because Pcyt1a undergoes reversible dissociation with membranes (Cornell and Northwood, 2000), it is possible that the overnight purification procedure used by Taoufiq et al. (2020), may have resulted in its dissociation from SV membranes. Alternatively, this discrepancy may have arisen during the computational assignment of detected peptides, as both our study and that of Taoufiq et al. (2020) also detected the closely related isoform Pcyt1b (Extended Data Figs. 2-1, 5-1). Finally, the top 500 most intense proteins also included the phospholipase A2 variant platelet-activating factor acetylhydrolase (Pla2g7), a secreted protein that metabolizes oxidized lipids and acts as an

Figure 5. Synaptic vesicles copurify with proteins involved in lipid homeostasis, including ApoE and the lipoprotein receptor Lrp1. Vesicles were prepared by immunoprecipitation and elution with the 1D4 peptide (rho1D4 mAb beads) or SDS (exo-syt1 mAb beads) and subjected to Orbitrap LC-MS (n = 2 biological replicates per purification method). A, Comparison of protein identifications among syt1-IP, rho1D4-IP with peptide elution, and SVs prepared by classical methods (Taoufiq et al., 2020). B, Scatter plot demonstrating robust correlation of intensity values for SVs purified by syt1-IP versus rho1D4-IP with peptide elution. All proteins with quantifiable intensity in at least one replicate were included. A small cluster of proteins enriched in Rho1D4-IP with peptide elution consists of ribosomal proteins that copurify with this method. C, Proteins with well-established roles in lipid transport and metabolism were manually identified from the top 500 most intense proteins in each condition. Closed dots represent values from individual replicates. ApoE predominated among the detected apolipoproteins and was among the top 80 most intense proteins in all replicates, regardless of purification method. Atp9a and Atp8a1 are phospholipid flippases that maintain physiologic distributions of phosphatidylserine. Pcyt1a regulates phosphatidylcholine abundance via synthesis of phosphocholine, a rate-limiting step in phosphatidylcholine synthesis. Monoglyceride lipase cleaves monoaoylglycerol molecules and plays key roles in endocannabinoid signaling. The fatty-acid-CoA ligase Acsbg1 activates long- and very-long-chain fatty acids with coenzyme A for downstream metabolic processing. Pla2g7 is a phospholipase A2 isozyme important for immune function and breakdown of oxidized lipids. Abundance values for canonical high-abundance SV proteins are given for comparison. Source data used to generate this figure are shown in Extended Data Figure 5-1, and a comparison of proteins detected exclusively by classical approaches (Taoufiq et al., 2020) or by immunopurification (this study) is shown in Extended Data Figure 5-2.
elution. Importantly, hybridomas for both the α-syt1 and α-SV2 mAbs used here are available from the Developmental Studies Hybridoma Bank (https://dshb.biology.uiowa.edu/), which should enable their production in the required quantities by independent investigators.

Our proteomics analyses (Fig. 2, Fig. 5, Extended Data Figs. 2-1, 5-1) identified several proteins with previously unestablished roles in SV lipid metabolism that may regulate numerous elements of SV function. The abundance of the phosphatidylserine (PS) flipase Atp8a1 (Hiraizumi et al., 2019) suggests that SVs actively maintain the appropriate polarity of PS, and the presence of the PC biosynthetic enzyme Pcyt1a suggests a regulatory mechanism to maintain the PC content of SV membranes (Wang et al., 2005). The conical shape of PS suggests that Atp8a1 may also drive the formation of highly curved SV membranes (Takada et al., 2018) alongside the proteins synapsin and synaptophysin (Park et al., 2021). SVs also contain substantial quantities of the enzyme long-chain-fatty-acid—CoA ligase ACSBG1 (Fig. 5, Extended Data Figs. 2-1, 5-1), also known as lipidosin, which enables an early step in the metabolism of long- and very-long-chain fatty acids (Min and Benzer, 1999; Pei et al., 2003; Sheng et al., 2009). In mammals, this protein is selectively expressed in brain and testis (Pei et al., 2003), and its abundance in SVs raises further questions about the roles of SVs in neuronal lipid metabolism. The presence of the phospholipase A2 variant platelet activating factor acetylhydrolase (Pla2g7), also known as lipoprotein-associated phospholipase A2, suggests a mechanism by which cycling of SVs might drive metabolism of oxidized lipids and modulate the local immunologic environment (Tjoelker et al., 1995; Tellis and Tselepis, 2009) or presynaptic Ca²⁺ (Hammond et al., 2016). We emphasize that any number of lipid-dependent processes, including membrane trafficking and endocannabinoid signaling, may be shaped by enzymes that regulate the distribution of fatty acids at nerve terminals. Selective ablation of these proteins in models of neurodevelopment, behavior, and synaptic transmission will help clarify their functional roles.

Our LC-MS results also raise provocative questions about the role of SV cycling in presynaptic lipid homeostasis and clearance of the Alzheimer’s disease–associated protein product amyloid-β (Aβ). Presynaptic dysfunction is an early hallmark of Alzheimer’s pathology (Terry et al., 1991; Hsia et al., 1999; Hark et al., 2021), but much remains unclear about the molecular interactions underlying this presynaptic vulnerability. Lipid transport systems likely play critical roles; ApoE, a major component of the lipoproteins that enable neuronal cholesterol uptake, is functionally associated with Alzheimer’s pathology (Corder et al., 1993; Li et al., 2012) and the clearance of extracellular Aβ (Strittmatter et al., 1993; Verghese et al., 2013). Molecular studies suggest cholesterol itself may drive protein aggregation at nerve terminals (Habchi et al., 2018). Moreover, the promiscuous endocytic receptor Lrp1 internalizes not only cholesterol-rich ApoE lipoproteins but also Aβ (Deane et al., 2004). However, although ApoE has been shown to accumulate in nerve terminals (Bilousova et al., 2019), and the SV cycle has been implicated as a key point of vulnerability in the early stages of Alzheimer’s pathology (Hark et al., 2021), a connection between ApoE and Aβ uptake and the SV cycle has not to our knowledge been established. Such a role is not unexpected; SVs are cholesterol-rich organelles (Takamori et al., 2006), and neurons normally rely on astrocyte-synthesized lipoproteins containing ApoE for cholesterol uptake (Vance et al., 1994; Mauch et al., 2001; Füünschilling et al., 2007; Nieweg et al., 2009).

Strikingly, our proteomics experiments on immunopurified SVs revealed both ApoE and Lrp1 in anti-syt1 and rho1D4 immunoprecipitates (Fig. 5, Extended Data Figs. 2-1, 5-1), implying that ApoE may traffic in nerve terminals via the SV cycle. We note that these proteins have been consistently detected in SVs regardless of purification method; α-syt1–immunoprecipitation and elution with SDS, rho1D4–immunoprecipitation and elution by peptide or SDS (Fig. 5), or classical methods (Takamori et al., 2006; Taoufiq et al., 2020). Given recent evidence that reactive astrocytes may secrete cytotoxic lipids via ApoE lipoproteins (Guttenplan et al., 2021), further studies may explore the links among presynaptic lipid signaling, lipoprotein trafficking, neuroinflammation, and the SV cycle.

We emphasize that the notion of a pure SV preparation is somewhat simplistic, as trafficking organelles in brain will have varying degrees of SV-like identity based on protein composition or physical characteristics. Most SV preparations likely includes small populations of early and late endosomes, lysosome-like vesicles, and organelar intermediates on a continuum from Golgi-derived SV precursor vesicles to mature SVs. For example, published SV proteomics studies (Chantranupong et al., 2020; Taoufiq et al., 2020) and the present study (Extended Data Figs. 2-1, 5-1) report the detection of lysosome-associated glycoproteins (LAMP1/2/5) at relatively low abundance in SV samples. As such, the presence of ApoE and Lrp1 in this preparation does not imply that ApoE traffics preferentially though the SV cycle—neither ApoE nor Lrp1 is limited to nerve terminals (Uhlen et al., 2015)—but our findings do suggest that some cotrafficking of ApoE–Lrp1 vesicles and SVs may occur. Although ApoE undergoes endocytic recycling (Heeren et al., 2001) and can be internalized and trafficked from distal axons (Amaratunga et al., 1996), its precise itinerary following endocytosis in the axon has not yet been established. ApoE may thus accumulate in SVs, which represent a uniquely vulnerable compartment (Hark et al., 2021), via SV recycling or other endosomal delivery pathways. In any case, the present results suggest further exploration of the SV cycle as a direct link among neuronal activity, lipid homeostasis, and proteostasis at the nerve terminal. Finally, our results suggest that modulation of synaptic activity or presynaptic membrane-trafficking processes, in addition to presynaptic lipid metabolism per se, may hold potential for the prevention or treatment of presynaptic neuropathological processes.

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