Molecular Characterization of AMPA-Receptor-Containing Vesicles

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Regulated delivery of AMPA receptors (AMPARs) to the postsynaptic membrane is an essential step in synaptic strength modification, and in particular, long-term potentiation (LTP). While LTP has been extensively studied using electrophysiology and light microscopy, several questions regarding the molecular mechanisms of AMPAR delivery via trafficking vesicles remain outstanding, including the gross molecular make up of AMPAR trafficking organelles and identification and location of calcium sensors required for SNARE complex-dependent membrane fusion of such trafficking vesicles with the plasma membrane. Here, we isolated AMPA-containing vesicles (ACVs) from whole mouse brains via immunoisolation and characterized them using immunoelectron microscopy, immunoblotting, and liquid chromatography–tandem mass spectrometry (LC–MS/MS). We identified several proteins on ACVs that were previously found to play a role in AMPAR trafficking, including synaptobrevin-2, Rabs, the SM protein Munc18-1, the calcium-sensor synaptotagmin-1, as well as several new candidates, including synaptophysin and synaptogyrin on ACV membranes. Additionally, we identified two populations of ACVs based on size and molecular composition: small-diameter, synaptobrevin-2- and GluA1-containing ACVs, and larger transferrin-receptor-, GluA1-, GluA2-, and GluA3-containing ACVs. The small-diameter population of ACVs may represent a fusion-capable population of vesicles due to the presence of synaptobrevin-2. Because the fusion of ACVs may be a requisite of LTP, this population could represent trafficking vesicles related to LTP.

Keywords: AMPAR trafficking, synaptic plasticity, proteomics, vesicle fusion, SNAREs

INTRODUCTION

At glutamatergic synapses, AMPA receptors (AMPARs) are responsible for the largest component of postsynaptic responses in the form of cation influx, and along with NMDARs, are major contributors to various forms of synaptic plasticity including long-term potentiation (LTP) (Dingledine et al., 1999; Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Collingridge et al., 2004; Shepherd and Huganir, 2007; Newpher and Ehlers, 2008). Upon the arrival of an action potential, glutamate is released from synaptic vesicles into the synaptic cleft where it binds to postsynaptic AMPARs. When bound with glutamate, AMPARs open, allowing cations to enter and
depolarize the postsynaptic cell. As a requisite of LTP (Malinow and Malenka, 2002), the cellular correlate of memory (Nabavi et al., 2014), AMPAR trafficking vesicles (ATVs) are exocytosed and AMPARs are recruited to the synapse, increasing the postsynaptic response (Lledo et al., 1998). The increased presence of AMPARs in the postsynaptic membrane has been characterized by light microscopy and electrophysiology studies, but little is known about the molecular composition of ATVs and the process by which they exocytose at the plasma membrane (Noel et al., 1999; Shi et al., 1999; Takumi et al., 1999; Liu and Cull-Candy, 2000; Passafaro et al., 2001; Ju et al., 2004). AMPA receptors at the synapse come from two sources: receptors that have been recycled from the plasma membrane and receptors that have been synthesized de novo. Regardless of etiology, AMPARs are trafficked in ATVs before they are inserted into the plasma membrane in a SNARE-dependent process (Jurado et al., 2013; Wu et al., 2017). While much is known about SNARE-dependent membrane fusion elsewhere in neurons (e.g., during neurotransmitter release via synaptic vesicle exocytosis), AMPAR insertion via ATVs has only recently beginning to be elucidated. The insertion of AMPARs during LTP is particularly intriguing due to evidence that the process is calcium-triggered and involves synaptotagmins (Wu et al., 2017). Electrophysiology studies revealed that syntaxin 3 (Stx-3), SNAP-47, and synaptobrevin 2 (Syb2) are SNARE proteins involved in ATV fusion during LTP and that synaptotagmin-1 (Syt1) and −7 (Syt7) are the calcium sensors for this process (Jurado et al., 2013; Wu et al., 2017). Rab proteins, including Rab5, Rab8, Rab11, and Rab39, and the transferrin receptor (TfR) also play a key role in AMPAR delivery to synapses (Gerges et al., 2004; Liu et al., 2016). Despite these discoveries, there are many outstanding questions surrounding the ATV lifecycle, from ATV fusion to AMPAR endocytosis. For example, the cellular localization of most synaptotagmins is unknown. While Syt1, a key synaptotagmin involved in synaptic vesicle fusion, and other synaptotagmins have been found on synaptic vesicles, it is not known whether synaptotagmins are likewise trafficked on ATVs. Moreover, it is unclear to what extent proteins are sorted as AMPARs are endocytosed, stored in recycling endosomes, and inserted back into the postsynaptic membrane.

Due to their small size, relatively low abundance (compared to synaptic vesicles), and relative transience in vivo, ATVs have been challenging to study (Kittler and Moss (eds), 2006). Electron microscopy studies have yet to uncover convincing evidence of ATVs at the synapse perhaps because deliveries of AMPARs to the postsynaptic membrane often happen after induction of synaptic plasticity. The transience of AMPAR delivery and the difficulty of specifically targeting synapses that are undergoing plasticity with electron microscopy makes studying the molecular components involved in AMPAR trafficking in situ challenging. Advances in organelle isolation from synaptosomes have made it possible to faithfully isolate small organelles, specifically synaptic vesicles, for molecular characterization (Ahmed et al., 2013). To overcome the problems associated with studying AMPAR trafficking in vivo, we have adopted a similar strategy to specifically isolate AMPA-containing vesicles (ACVs) from synaptosomes purified from whole mouse brains. Subcellular fractions were purified from neurons using multiple rounds of differential centrifugation, after which AMPAR-containing components were immunoprecipitated with a GluA1 antibody and then isolated by specific elution with a peptide that competes with the GluA1 subunit of AMPARs. The resulting sample was characterized using immunoblotting, liquid chromatography–tandem mass spectrometry (LC–MS/MS), and immunoelectron microscopy. Here, we offer the first unbiased characterization of GluA1-containing ACVs. LC–MS/MS confirms several previously identified proteins found to be involved in AMPAR trafficking and identifies potential new candidates for AMPAR receptor trafficking. Immunoelectron microscopy reveals heterogeneous populations of ACVs in terms of protein compositions and vesicle diameters. Combined, these data offer an unbiased candidate list of proteins potentially involved in AMPAR receptor trafficking.

**MATERIALS AND METHODS**

**Animal Ethics Statement**

The animal study was reviewed and approved by the Administrative Panel on Laboratory Animal Care (APLAC) at Stanford University (IACUC #29981).

**Purification of AMPA-Containing Vesicles**

To isolate ACVs, we followed a previously developed protocol for synaptosome generation and synaptic vesicle isolation (Ahmed et al., 2013) and extensively modified it to specifically purify ACVs. Eight to twelve P20 CD-1 mice were anesthetized using isoflurane in an open-drop chamber, and whole brains were immediately removed and homogenized. (See Figure 1 for full summary). This initial homogenate was spun in a JA-20 rotor at 2700 RPM (880 G) for 10 min to pellet blood vessels and other large cellular debris. The supernatant was then spun at 10,000 RPM (12,064 G) for 15 min to pellet synaptosomes. The supernatant was discarded and the periphery of the pellet was resuspended, which helps to remove mitochondria, before spinning at 11,000 RPM (14,597 G) for 15 min. The supernatant was again discarded, and the pellet resuspended to 5 ml total volume. The suspension was added to a Dounce homogenizer along with 45 ml of ultrapure water and was briefly homogenized to hypoosmotically lyse the synaptosomes. Immediately afterward, 60 µl of 1 mg/ml pepstatin A and 120 µl of 200 mM PMSF in 1 M HEPES was added. This solution was spun at 19,500 RPM (45,871 G) for 20 min to pellet plasma membrane and large cellular debris while leaving small organelles like vesicles in solution (LP1 for “lysis pellet 1”). The supernatant was then removed and spun in a Ti-70 ultracentrifuge at 50,000 RPM (256,631 G) for 2 h at 4°C to pellet small organelles like trafficking vesicles (LP2 for “lysis pellet 2”). The LP2 pellet was transferred to a small homogenizer and resuspended in 2 ml of PBS by homogenization and mechanically sheared through a 27-gauge needle. The concentration of LP2 was determined using BCA and aliquoted into 2 mg aliquots at approximately 5 µg/µl. Any LP2 not used immediately for ACV isolation was flash frozen with liquid nitrogen and stored at −80°C until use.
To isolate ACVs from LP2, 1 aliquot of 2 mg LP2 was diluted to 1 ml total volume in 0.5% BSA in PBS, 5 µl of mouse anti-GluA1 monoclonal antibody (1 µg/µl, Synaptic Systems, Göttingen, Germany) was added and allowed to bind while rotating for 12 h at 4°C. To prevent non-specific binding, 50 µl of paramagnetic protein G beads (Dynabeads, ThermoFisher Scientific, Waltham, MA, United States) were washed three times in 0.5% BSA in PBS for 15 min on ice and then three times in PBS for 5-min washes on ice prior to addition of LP2. The LP2 mixture was then added to the beads and rotated for 2 h at 4°C. Dynabeads
were separated from solution using a magnet, and the flow through was collected for western blot analysis. ACVs were then gently eluted with three, 20-min washes with 33 µl of GluA1 peptide (20 µg/µl) representing the same synthetic peptide the antibody was created against (sequence: SHSSGMPLGATGL) (GenScript Biotech, Piscataway, NJ, United States). ACVs were then immediately used and continually stored on ice at 4°C. Protein concentration was measured by Bradford assay. Serial dilutions of BSA were used to generate a standard curve.

GluA1 Knockout Mice
Knockout mutant mice for GRIA1, the gene encoding GluA1, have been previously described (Zamanillo et al., 1999). Knockout mice were generated by interbreeding heterozygous mice. The same immunoisolation protocol was used as for wild-type mice.

Western Blots
For western blot analysis, samples were first separated by SDS-PAGE and then electrophoretically transferred onto membranes. After transfer, the membranes were then treated with blocking buffer and labeled using an iBind Flex (ThermoFisher Scientific). GluA1 (Abcam – ab1504, rabbit, 1:2,000, Cambridge, United Kingdom), GluN1 (Spectronics Systems – 114-003, rabbit, 1:1,000), PSD-95 (Abcam – ab18258, rabbit, 1:2,000), VGLUT1 (Abcam – ab77822, rabbit, 1:1,000), Lamp1 (Proteintech – 21997-1-AP, rabbit, 1:2,000, Rosemont, IL, United States), and golgin (Abcam – 84380, rabbit, 1:2,000) were each individually probed. A goat-anti rabbit secondary antibody conjugated with HRP was used for all chemiluminescent western blots (Abcam – ab672, 1:50,000), and a goat-anti rabbit secondary antibody conjugated with IRDye 800CW was used for all fluorescent western blots (Abcam – ab7672, 1:50,000). The bands were visualized either by immunofluorescence with a LI-COR Odyssey (Lincoln, NE, United States) or with chemiluminescence with a Konica Minolta – SRX101A (Tokyo, Japan). All antibodies were diluted from 1 mg/ml stock.

Transmission Electron Microscopy
Negative stain transmission electron microscopy (TEM) was performed on ACVs. Copper mesh grids were glow discharged in argon gas for 20 s before 4 µl of ACV eluate was applied and allowed to settle for 30 min. The grid was then washed three times with ultra-pure water. The grid was negatively stained using 1% uranyl acetate for 2 min then blotted and allowed to dry at room temperature for 20 min. The grid was imaged using a JEOL 1400 TEM at 120 keV. The diameters of ACVs were measured using ImageJ. Two diameters were measured using the line segment tool in ImageJ for each ACV; each measurement was scaled using the scale bar as reference for each given image. The two diameters were averaged together to get a final diameter. Immunogold labeling was performed for GluA2 (BioLegend, San Diego, CA, United States), GluA3 (Spectronics Systems), Syb2 (Abcam), Syt1 (Abcam), IRB (ThermoFisher Scientific), and Syp1 (Spectronics Systems). For immunogold labeling, the same protocol for negative stained TEM was performed; however, after ACV addition, the grids were incubated in a 1:50 dilution of rabbit polyclonal primary antibody in blocking buffer (0.5% BSA, 0.5% ovalbumin in PBS) for 1 h. Then three, 5-min washes in PBST were performed followed by a 1-h incubation in 1:50 10 nm gold goat anti-rabbit secondary antibody (Electron Microscopy Sciences – 25108, Hatfield, PA, United States). Three more 5-min washes in PBST were performed, and then samples were fixed in 8% glutaraldehyde for 30 s. Staining and imaging were performed as previously described.

Liquid Chromatography–Mass Spectrometry
Purified ACVs were resuspended in 50 µl 0.2% Rapigest (Waters, Milford, MA, United States) in 20 mM NH4HCO3 in 0.65 ml low protein binding polypropylene tubes before the addition of 5 mM DTT and incubation at 60°C for 30 min. After this, iodoacetamide was added to a final concentration of 7.5 mM and samples were incubated for 30 additional minutes. Samples were then digested with 2.5 µg of sequencing grade trypsin (Trypsin Gold, Mass spectrometry grade, Promega, Madison, WI, United States) at 37°C, overnight. A second aliquot of trypsin (1.5 µg) was added, and the samples incubated for an additional 3 h at 37°C. After this, samples were acidified by adding 5% formic acid and incubated for 30 min at room temperature. Tryptic peptides were recovered from the supernatant by C18 solid phase extraction using ZipTips (MilliporeSigma, Burlington, MA, United States), eluted in two, 7 µl drops of 50% acetonitrile and 0.1% formic acid, and evaporated and resuspended in 5 µl 0.1% formic acid for LC–MS/MS analysis.

Peptides resulting from trypsinization were analyzed on a QExactive Plus mass spectrometer (ThermoFisher Scientific) connected to a NanoAcquity Ultra Performance UPLC system (Waters). A 15-cm EasySpray C18 column (ThermoFisher Scientific) was used to resolve peptides (60-min 2–30% B gradient with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B, at a flow rate of 300 nl/min). MS was operated in data-dependent mode to automatically switch between MS and MS/MS. MS spectra were acquired between 350 and 1,500 m/z with a resolution of 70,000. For each MS spectrum, the top 10 precursor ions with a charge state of 2+ or higher were fragmented by higher-energy collision dissociation. A dynamic exclusion window was applied which prevented the same m/z from being selected for 10 s after its acquisition.

Peak lists were generated using PAVA in-house software (Guan et al., 2011). All generated peak lists were searched against the mouse subset of the UniProtKB database (SwissProt.2013.6.17) (plus the corresponding randomized sequences to calculate false discovery rate on the searches), using Protein Prospector (Clauser et al., 1999). The database search was performed with the following parameters: a mass tolerance of 20 ppm for precursor masses and 30 ppm for MS/MS, cysteine carboxamidomethylation as a fixed modification, and acetylation of the N terminus of the protein, pyroglutamate formation from N terminal glutamine, and oxidation of methionine as variable modifications. A 1% false discovery rate was permitted at the protein and peptide level. All spectra identified as matches to peptides of a given protein were
reported, and the number of spectra (peptide spectral matches, PSMs) was used for label free quantitation of protein abundance in the samples. Abundance index for each protein was calculated as the ratio of PSMs for a protein to the total PSMs for all components identified in the run divided by the polypeptide molecular weight.

**Additional Statistics**

The Kolmogorov–Smirnov test was performed to test statistical significance between an independent population of vesicles from the gross population of all ACVs isolated (Figure 1D, from three independent immunoisolations) and vesicles positively labeled with gold-conjugated antibodies against Syb2 ($p = 0.0101$, three immunoisolations), Syt1 ($p = 0.9382$, three immunoisolations), Syp1 ($p < 0.0001$, four immunoisolations), TIR ($p = 0.0100$, two immunoisolations), GluA2 ($p < 0.0001$, three immunoisolations), and GluA3 ($p < 0.0001$ three immunoisolations).

**RESULTS**

**AMPA-Containing Vesicle Isolation From Whole Mouse Brains**

To characterize the molecular composition of ACVs, synaptosomes were purified from whole brains of 6–12 P20 mice and hypoosmotically lysed to release their contents (Ahmed et al., 2013). The resulting lysis pellet (LP2), comprised of synaptosome contents, was flash frozen and stored at −80°C until used. GluA1-containing components were first extracted from LP2 using an anti-GluA1 antibody (Figure 1A). Antibody was allowed to bind overnight at 4°C and was subsequently bound to protein G paramagnetic beads before ACVs were gently eluted by competing with a peptide that contains the GluA1 antibody epitope to allow for specific elution and isolation. As such, this elution is based on competition between GluA1 and the peptide which is present in large molar excess. Thus, contaminants that do not specifically bind to the antibody recognition site, should remain on the beads. Western blot analysis confirmed the presence of GluA1 in LP2 and the eluate (Figure 1B and Supplementary Figure 1). Additionally, western blot analysis confirmed the presence of VGLUT1, a marker of glutamatergic synaptic vesicles (a potential contaminant), in LP2 but not in the eluate. Negative stain electron micrographs (Figure 1C and Supplementary Figure 2) revealed that the purification yielded vesicles with a diameter of 102.7 ± 50.8 nm (arithmetic mean) (Figure 1D), marking the first time ACVs were visualized. To further confirm the fidelity of the ACV preparation, the same immunoprecipitation and GluA1 peptide elution protocol was performed using LP2 purified from GLUA1−/− knockout mice. Western blot analysis confirmed the deletion of GLUA1 but the retention of VGLUT1 expression (Figure 1B). There were substantially fewer vesicles identified in the sample isolated from knockout animals as assessed by negative stain electron microscopy (Figure 1E and Supplementary Figures 2, 3): Immunoeisolation from wild-type mice yielded 4.83 vesicles/µm² (5 micrographs, 535 vesicles in 110.8 µm²), while immunoeisolation from knockout mice yielded 0.30 vesicles/µm² (5 micrographs, 34 vesicles in 112.0 µm²) (for all images, see Source Data); note that the same amount of protein (as assessed by BCA) was inputted into the same immunoeisolation step for both wild-type and knockout preparations. Additionally, we measured the total protein concentration in the elution by Bradford assay and found the wild-type eluate contained ∼35.3 µg/ml compared to GLUA1−/− knockout eluate which contained only ∼5.8 µg/ml. It is important to note that due to the size and amino acid composition of the elution peptide, the elution peptide itself does not provide a detectable signal in the Bradford assay. For both wild-type and knockout preparations, defined aliquots of 2 mg of total protein LP2 were inputted into the same immunoeisolation procedure, so the decreased yield from immunoeisolation from the knockout LP2 is indicative of a decreased amount of GluA1-containing material. Therefore, our immunoeisolation procedure targets ACVs (including ATVs) and minimizes contamination by other components.

**Immunoelectron Microscopy Revealed Molecular Components of AMPA-Containing Vesicles**

Immunoelectron microscopy was performed on the isolated ACVs to assess the frequency of protein localization on ACVs for several known AMPAR-associated proteins (Figures 2, 3). Secondary antibody concentration was optimized to minimize non-specific, background gold (<1 free gold per field of view). A positive hit was defined as a gold particle within 5 nm of an ACV. AMPAR subunits GluA2 and GluA3 were probed to...
test for the presence of these subunits in the GluA1-affinity purified ACVs. GluA2 was found on 42.6% of ACVs, and the GluA3 subunit was found on 36.7% of ACVs. TfR, a known marker of AMPAR endosomes, was identified on 48.2% of ACVs. Synaptophysin 1 (Syp1) was identified on 90.2% of vesicles. Syb2 was found 82.1% of ACVs, while Syt1 was identified on 44.0% of ACVs. Additionally, the diameters of ACVs that were labeled by GluA2 (140.1 ± 52.5 nm), GluA3 (134.5 ± 60.2 nm), TfR (121.7 ± 66.4 nm), Syp1 (116.2 ± 50.8 nm), Syb2 (93.9 ± 43.8 nm), and Syt1 (105.2 ± 54.6 nm) were measured (all arithmetic means) (Figure 2H). As a negative control, VGLUT1 (vesicular glutamate transporter), a marker of glutamatergic synaptic vesicles, was probed (data not shown), and only 8.4% of ACVs were positive for VGLUT1. The Kolmogorov–Smirnov test was performed, comparing the cumulative frequency distribution for each marker to the overall population of ACVs obtained from the negative stain experiments shown in Figure 1D (Figure 2I). The cumulative frequency distribution for Syb2-labeled ACVs was significantly shifted to the left, indicating smaller diameters (p = 0.0101), while the Syp1 (p < 0.0001), TfR (p = 0.0100), GluA2 (p < 0.0001), and GluA3 (p < 0.0001) distributions were significantly shifted to the right (larger diameters). Syt1 was not significantly shifted from the global ACV diameter distribution (p = 0.9382). Smaller, Syb2-labeled vesicles are unlikely to be synaptic vesicles due to the low frequency of VGLUT1-labeled vesicles and the substantial difference in size between Syb2-labeled vesicles and the 40-45 nm diameter that has previously been reported for synaptic vesicles (Takamori et al., 2006). Additionally, the mean diameter of VGLUT1-labeled vesicles (arithmetic mean of 97.6 ± 54.8 nm) is also much larger than the reported diameters of synaptic vesicles, which suggests that the small population of VGLUT1-labeled vesicles are most likely small endosomes or membrane fragments.
Liquid Chromatography-Tandem Mass Spectrometry Analysis Identifies Known AMPAR Receptor Trafficking Proteins and Candidates for New Proteins

Liquid chromatography–tandem mass spectrometry was performed on isolated ACVs. We identified a total of 755 unique proteins with expectation values <0.005 across three biological replicates (Fenyö and Beavis, 2003). We applied two additional filters to these 755 proteins to ensure high quality and abundance. Of those 755 unique proteins, 442 proteins were identified in two or more data sets (Figure 4A). The sequence coverage (fraction of protein sequence that was identified) for 180 proteins was greater than 7.5%, suggestive of higher abundance. Proteins were manually categorized based on function and cellular localization (Figure 4B). Cytosolic proteins, channels/transporters, and Rabs were the most commonly identified protein classes with 39, 23, and 21 hits, respectively. Among the top proteins enriched in ACVs (Table 1) are AMPAR subunits GluA1, GluA2, and GluA3, as well as AMPAR-associated Dnajc13 (Perrett et al., 2015), TIR (Liu et al., 2016), neuroplastin (Jiang et al., 2021), and ABHD6 (Wei et al., 2016). In addition, the genes for Rab5, 8, 11, and 39, all implicated in AMPAR trafficking, were also among the top 180 candidates (Gerges et al., 2004). Furthermore, other synaptic proteins that have yet to be identified as AMPAR-trafficking-associated, including Sypl, synaptogyrin-1 (Syngr1), and −3 (Syngr3), and Munc18-1, were identified (Table 1).

**DISCUSSION**

**AMPA-Containing Vesicles Can Be Specifically Purified From Whole Mouse Brains**

Due to their relatively low abundance at synapses compared to other synaptic content (e.g., synaptic vesicles), ATVs have been difficult to characterize in the past. Here, we developed
a protocol to specifically purify and enrich ACVs from synaptosome lysate via immunoisolation using a monoclonal anti-GluA1 antibody. A key methodological advance compared to previous isolation protocols consists of specific, competitive, elution off the paramagnetic beads using a molar excess of a small peptide that corresponds to the epitope of the GluA1 monoclonal antibody (Figure 1A). While previous approaches more typically used harsh elution conditions to shear or denature all bound components from the beads, our specific and gentle elution method with the GluA1 epitope peptide minimizes contamination by non-specifically bead-bound components. Indeed, when applied to GluA1 KO mice, our immunoisolation method yielded substantially less vesicular material (Figures 1C,E and Supplementary Figures 2, 3), despite the same amount of LP2 input, providing further evidence that immunoisolation via peptide elution is specific. Such little material was generated from immunoisolation from GluA1 KO mice LP2 that LC–MS/MS experiments would require impractically large amounts of starting material. Western blot analysis of samples taken from steps in the purification process further supports the specificity of this isolation. Seven cellular components were probed by western blot (Figure 2A). GluA1, the AMPAR subunit being enriched, was present in each step of the purification process and was enriched in the final elution. In contrast, GluN1 (NMDA receptor subunit), PSD-95 (postsynaptic density component), LAMP1 (late endosome component), and golgin (Golgi marker) were all present throughout the purification process but did not bind to beads nor appear in the final eluate. Typically, synaptosomes generated via differential centrifugation have primarily been used to study presynaptic components. Our results suggest that synaptosomes present in the crude synaptosome fraction (P3) also preserve postsynaptic components (GluA1, GluN1, and PSD-95) and that these postsynaptic components are also present in LP2, the input fraction for immunoprecipitation and GluA1 peptide elution. Therefore, the described ultracentrifugation protocol generated a fraction containing relevant postsynaptic components. In the subsequent elution step with GluA1 peptide, the postsynaptic GluN1 and PSD-95 components were removed, resulting in GluA1 components that should include ATVs. Although we cannot rule out that some postsynaptic plasma membrane components are present in our isolation, the absence of these other postsynaptic residents strongly argues for the specificity and purity of our sample. Likewise, we cannot rule out that a fraction of the isolated ACVs are pre-synaptic components. However, our synaptosome preparation likely preserves some postsynaptic ACVs, considering that LTP can be induced in synaptosomes (Corera et al., 2009) and that AMPAR subunits are synthesized in isolated synaptosomes (Maghsoodi et al., 2008). Thus, at least some of the isolated ACVs should be ATVs.

Immunoelectron microscopy analysis further confirmed the specificity of ACV purification. Unsurprisingly, Syb2, a SNARE protein essential for AMPAR insertion during LTP, labeled 82.1% of ACVs (Jurado et al., 2013). In addition, 42.6% of ACVs were positive for the GluA2 subunit of the AMPAR. This aligns well with evidence that GluA1/GluA2 heteromers are the most common AMPAR composition (Lu et al., 2009; Zhao et al., 2019). Furthermore, 36.7% of ACVs were positive for the GluA3 subunit. This could perhaps be reflective of GluA1/A3 heteromers; it has been previously observed that ~10% of GluA3-containing AMPARs also contain GluA1 (Wenthold et al., 1996; Diering and Huganir, 2018). Alternatively, multiple AMPARs could be contained in the same ACV, and this observation could be reflective of GluA2/A3 heteromers.

Substantial contamination from synaptic vesicles in our preparation is unlikely for several reasons. First, VGLUT1 was not present in the final eluate, as measured by western blot (Figure 1B) and only a small fraction of purified vesicles was positive for VGLUT1 via immunoelectron microscopy (Figure 2G). Second, the purified vesicle population with
TABLE 1 | Protein ontology of proteins in ACVs identified with LC–MS/MS using gene ontology resource.

| Gene name | Protein name |
|-----------|--------------|
| **AMPA subunit** | |
| GRIA1 | Glutamate receptor, ionotropic, AMPA1 (alpha 1) |
| GRIA2 | Glutamate receptor, ionotropic, AMPA2 (alpha 2) |
| GRIA4 | Glutamate receptor, ionotropic, AMPA4 (alpha 4) |
| **Calcium sensor** | |
| SYT1 | Synaptotagmin I |
| SYT2 | Synaptotagmin II |
| **Cell adhesion** | |
| BSG | Basigin |
| NCAM1 | Neural cell adhesion molecule 1 |
| NEGR1 | Neuronal growth regulator 1 |
| NPTN | Neuroplastin |
| THY1 | Thymus cell antigen 1, theta |
| **Channel/transporter** | |
| ATP1A1 | ATPase, Na+/K+ transporting, alpha 1 polypeptide |
| ATP1A2 | ATPase, Na+/K+ transporting, alpha 2 polypeptide |
| ATP1A3 | ATPase, Na+/K+ transporting, alpha 3 polypeptide |
| ATP1B1 | ATPase, Na+/K+ transporting, beta 1 polypeptide |
| ATP1B2 | ATPase, Na+/K+ transporting, beta 2 polypeptide |
| ATP1B3 | ATPase, Na+/K+ transporting, beta 3 polypeptide |
| ATP2A2 | ATPase, Ca++ transporting, cardiac muscle, slow twitch 2 |
| ATP2B1 | ATPase, Ca++ transporting, plasma membrane 1 |
| ATP2B2 | ATPase, Ca++ transporting, plasma membrane 2 |
| ATP2B3 | ATPase, Ca++ transporting, plasma membrane 3 |
| ATP2B4 | ATPase, Ca++ transporting, plasma membrane 4 |
| ATP6V0A1 | ATPase, H+ transporting, lysosomal V0 subunit A1 |
| ATP6V0D1 | ATPase, H+ transporting, lysosomal V0 subunit D1 |
| ATP6V1A | ATPase, H+ transporting, lysosomal V1 subunit A |
| ATP6V1B2 | ATPase, H+ transporting, lysosomal V1 subunit B2 |
| ATP8A1 | ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1 |
| SLC12A5 | Solute carrier family 12, member 5 |
| SLC17A6 | Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6 |
| SLC17A7 | Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7 |
| SLC32A1 | Solute carrier family 32 (GABA vesicular transporter), member 1 |
| SLC6A17 | Solute carrier family 6 (neurotransmitter transporter), member 17 |
| VDAC1 | Voltage-dependent anion channel 1 |
| VDAC3 | Voltage-dependent anion channel 3 |
| **Cytosolic** | |
| ABHD6 | Abhydrolase domain containing 6 |
| ACSL6 | Acyl-CoA synthetase long-chain family member 6 |
| ACTB | Actin, beta |
| ADRBK2 | G protein-coupled receptor kinase 3 |
| AK5 | Adenylate kinase 5 |
| ALG2 | Asparagine-linked glycosylation 2 (alpha-1,3-mannosyltransferase) |
| AP2A1 | Adaptor-related protein complex 2, alpha 1 subunit |
| AP2A2 | Adaptor-related protein complex 2, alpha 2 subunit |
| AP2M1 | Adaptor-related protein complex 2, mu 1 subunit |

(Continued)
TABLE 1 | (Continued)

| Gene name | Protein name |
|-----------|--------------|
| SFPQ      | Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated) |
| TRPS3I11  | Transformation related protein S3 inducible protein 11 |
| **Other** |                                         |
| PLP1      | Proteolipid protein (myelin) 1 |
| PRSS1     | Protease, serine 1 (trypsin 1) |
| SRSF3     | Serine and arginine-rich splicing factor 3 |
| TARDBP    | TAR DNA binding protein |
| U2AF1     | U2 small nuclear ribonucleoprotein auxiliary factor (U2AF) 1 |
| **Protein folding** |                                      |
| CANX      | Calnexin |
| HSP90B1   | Heat shock protein 90, beta (Grp94), member I |
| HSPA5     | Heat shock protein 5 |
| HSPA8     | Heat shock protein 8 |
| PDI3      | Protein disulfide isomerase associated 3 |
| TMX2      | Thioredoxin-related transmembrane protein 2 |
| VMA21     | VMA21 vacuolar H+-ATPase homolog (S. cerevisiae) |
| **Rabs**  |                                         |
| RAB1      | Ribonuclease, RNase A family 4 |
| RAB10     | RAB10, member RAS oncogene family |
| RAB11B    | RAB11B, member RAS oncogene family |
| RAB13     | RAB13, member RAS oncogene family |
| RAB14     | RAB14, member RAS oncogene family |
| RAB15     | RAB15, member RAS oncogene family |
| RAB18     | RAB18, member RAS oncogene family |
| RAB1A     | RAB1A, member RAS oncogene family |
| RAB1B     | RAB1B, member RAS oncogene family |
| RAB2A     | RAB2A, member RAS oncogene family |
| RAB3A     | RAB3A, member RAS oncogene family |
| RAB3B     | RAB3B, member RAS oncogene family |
| RAB3B     | RAB3B, member RAS oncogene family |
| RAB3C     | RAB3C, member RAS oncogene family |
| RAB5A     | RAB5A, member RAS oncogene family |
| RAB6A     | RAB6A, member RAS oncogene family |
| RAB6B     | RAB6B, member RAS oncogene family |
| RAB7A     | RAB7A, member RAS oncogene family |
| RAB8A     | RAB8A, member RAS oncogene family |
| RAB8B     | RAB8B, member RAS oncogene family |
| **Receptor** |                                         |
| GNA1I     | Guanine nucleotide binding protein (G protein), alpha inhibiting 1 |
| GNA2I     | Guanine nucleotide binding protein (G protein), alpha inhibiting 2 |
| GNAO1     | Guanine nucleotide binding protein, alpha O |
| GNAQ      | Guanine nucleotide binding protein, alpha q polypeptide |
| GNBP1     | Guanine nucleotide binding protein (G protein), beta 1 |
| GNBP2     | Guanine nucleotide binding protein (G protein), beta 2 |
| LP1P1     | Low density lipoprotein receptor-related protein 1 |
| M6PR      | Mannose-6-phosphate receptor, cation dependent |
| P2RY12    | Purinergic receptor P2Y, G-protein coupled 12 |
| PRGC1     | Progesterone receptor membrane component 1 |
| SORT1     | Sortilin 1 |
| TFRC      | Transferrin receptor |

(Continued)
less than 50 nm (Figure 1D). Third, the purified vesicles were positive for several markers in immunoelectron microscopy that are unlikely to be in synaptic vesicles, including TIR, GluA2, and GluA3 (Figure 2G). Furthermore, immunooisolation from GluA1 knockout mice yielded negligible material. The presence of synaptophysin, typically thought of as a synaptic vesicle marker, on 90.2% of vesicles isolated from wild-type mice is more likely suggestive of the presence of synaptophysin on ATVs as opposed to contamination due to synaptic vesicles. Therefore, our ACV preparation is relatively pure and contains key proteins associated with AMPAR delivery.

Liquid chromatography–tandem mass spectrometry also provided supportive evidence that ACV purification is specific. The GluA1, GluA2, and GluA4 subunits were all identified in the top mass spectrometry hits. GluA3 was also identified but had lower sequence coverage, possibly due to sequence similarity between it and other AMPAR subunits. Several of the top hits identified via mass spectrometry were Rab proteins, including Rab5, Rab8, Rab11, and Rab39, all of which are required for AMPAR trafficking (Gerges et al., 2004), and Rab5 (Brown et al., 2005; Hoogenraad et al., 2010), Rab8 (Gerges et al., 2004), and Rab11 (Hoogenraad et al., 2010) all of which localize in the postsynaptic terminal. Rab39 contributes to AMPAR trafficking from the endoplasmic reticulum to the Golgi, and mutations in this protein have been connected to autism spectrum disorders (Mignogna et al., 2015). Rab5 is required for AMPAR endocytosis (Brown et al., 2005), while Rab8 and Rab11 (Brown et al., 2007) are likely involved in AMPAR insertion into the plasma membrane. Mass spectrometry also identified several other proteins associated with AMPAR trafficking, including Lrp1 (Gan et al., 2014), TIR (Liu et al., 2016), Dnajc13 (Perrett et al., 2015), TDP-43 (Schwenk et al., 2016), and ABHD6 (Wei et al., 2016). Furthermore, Lrp1 (Gan et al., 2014) and TIR (Liu et al., 2016) colocalize with AMPARs. Additionally, NSF, AP-2, POR, ABHD6, and SACM1L have direct interactors of AMPARs (Schwenk et al., 2012; Shanks et al., 2012).

**Composition of AMPA-Containing Vesicles**

Immunoelectron microscopy combined with vesicle diameter analysis identified at least two possible unique populations of ACVs. Specifically, the cumulative frequency distribution of the average diameters of ACVs labeled with TIR was significantly shifted to larger diameters compared to an independent overall average of ACVs, while the cumulative frequency distribution of the average diameters of ACVs labeled with Syb2 was significantly shifted to smaller diameters (Figure 2I). These larger ACVs were also more likely to contain GluA2 and GluA3. Thus, our GluA1 immunooisolation whole mouse brain isolates at least two populations of vesicles (Figures 2H,1). The smaller-diameter population of vesicles likely represents a fusion-capable population of vesicles due to the prevalence of Syb2, while Syb2 was rarely observed associated with large ACVs. The fusion of Syb2-positive, GluA1-positive ATVs may play a role in LTP (Jurado et al., 2013). Therefore, the small-diameter Syb2-positive, GluA1-positive population of vesicles that we observe may represent ATVs essential for LTP. The larger ACVs containing TIR and a mixed population of AMPAR subunits may represent recycling endosomes.

Liquid chromatography–tandem mass spectrometry of GluA1 immunooisolated samples identified many of the SNARE and SNARE effector proteins involved in AMPAR insertion during LTP (Figure 4 and Table 1), including Stx-3, SNAP-47, and Syb2 (Jurado et al., 2013). Additionally, the N-terminal, Sec1/Munc18-like-binding portion of Stx-3 is essential for LTP (Jurado et al., 2013), providing evidence for the possible role of Munc18 in AMPAR insertion. Munc18-1 is associated with ACVs as observed by mass spectrometry, and combined with evidence that Munc18-1 binds to Stx-3 (Hata and Südhof, 1995), Munc18-1 is a likely candidate for a regulator of AMPAR insertion. In synaptic vesicle fusion, Munc18 stabilizes syntaxin-1A (Südhof, 2013), and Munc13 is required to aid in the transition of the syntaxin/Munc18 complex into the ternary trans-SNARE complex, a critical step to ensure parallel assembly of all SNARE complex components (Ma et al., 2013; Lai et al., 2017; Brunger et al., 2019). After fusion, the ternary SNARE complex is disassembled with the ATPase, NSF, and adaptor protein, SNAP, for use in future fusion events (Söllner et al., 1993; Mayer et al., 1996; Hanson et al., 1997). Therefore, Munc18, Munc13, NSF, and SNAP could also play roles in regulating SNARE assembly and disassembly during ATV fusion. Additionally, LC-MS/MS identified synaptotagmin-2 (Syt2), a calcium sensor that performs equivalent functions to Syt1 (Pang et al., 2006). Only 44.0% of ACVs contained Syt1 (Figure 2G), consistent with the implication of alternative calcium sensors such as Syt2 or Syt7 for AMPAR insertion (Wu et al., 2017). Furthermore, it is worth noting that the exact location of AMPAR insertion is an active area of exploration (Choquet and Hosy, 2020).

**Potential New AMPAR Trafficking Candidates**

Liquid chromatography–tandem mass spectrometry and immunoelectron microscopy of GluA1 immunooisolated ACVs revealed several potential new candidates with connections to AMPAR trafficking and neurological disease (Figures 2, 3 and Table 1). Syph1, best known as a synaptic vesicle marker, densely labeled ACVs, and Syph1, Syngr1, and Syngr3 were identified among the top mass spectrometry hits. Previously, synaptophysin and synaptogyrin have been shown to cooperatively contribute to LTP (Janz et al., 1999). Furthermore, Syngr3 may play a role in tauopathies, and the reduction of Syngr3 expression in neurons rescues synaptic plasticity deficits induced by tau (Largo-Barrientos et al., 2021). While important roles for synaptophysin and synaptogyrin have already been confirmed in the presynaptic terminal, the potential for a postsynaptic contribution has yet to be explored. We validated the presence of Syph1 on ACVs (Figures 2C,G), but further studies are needed to quantify the frequency of Syngr1 and Syngr3 on ACVs.

**Connections to Disease**

Many of the candidates identified LC-MS/MS of GluA1 immunooisolated ACVs have been implicated in neurological
The molecular characterization of ACVs presented here is the first time ACVs have been isolated and characterized. Our findings are a potential steppingstone in the understanding of neurological disorders. The molecular interactors for AMPARs and establish a framework for future AMPAR studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories as source data. The names of the repository/repositories and accession number(s) can be found below: https://datadryad.org/stash, https://doi.org/10.5061/dryad.r2280gbdh, https://doi.org/10.5061/dryad.jdfn2z3bd.

ETHICS STATEMENT

The animal study was reviewed and approved by the Administrative Panel on Laboratory Animal Care (APLAC) at Stanford University (IACUC #29981).

AUTHOR CONTRIBUTIONS

JP: conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, writing – original draft, and writing – review and editing. JO-P: formal analysis, validation, investigation, methodology, and writing – review and editing. ALB: supervision. ATB: conceptualization, supervision, funding acquisition, project administration, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2021.754631/full#supplementary-material

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