Published in final edited form as:

J Neurosci Methods. 2018 September 01; 307: 210–220. doi:10.1016/j.jneumeth.2018.05.022.

An optimized method for purification of whole brain-derived extracellular vesicles reveals insight into neurodegenerative processes in a mouse model of Alzheimer's disease.

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Abstract

Background: Alzheimer's disease (AD) is the major cause of dementia in older adults, and has increased dramatically in prevalence over the past several decades. Yet many questions still surround the etiology of AD. Recently, extracellular vesicles (EVs) that transport protein, lipid, and nucleic acids from cell to cell have been implicated in the clearance and propagation of misfolded proteins. Investigation of EVs in AD progression, and their potential diagnostic utility may contribute to understanding and treating AD. However, the challenges of isolating brain-derived EVs have in part hindered these studies.

New method: Here, we provide an optimized method for the purification of brain-derived EVs by iodixanol floatation density gradient for mass spectrometry analysis.

Results: We demonstrate the purity of these vesicles and the enrichment of EV proteins compared to sedimentation gradient isolation of vesicles. Moreover, comparative proteomic analysis of brain-derived EVs from healthy and AD mouse brains revealed differences in vesicular content including proteins involved in aging, immune response, and oxidation-reduction maintenance. These changes provide insight into AD-associated neurodegeneration and potential biomarkers of AD for early disease prediction.

Comparison with existing methods: Recent techniques have used sedimentation sucrose gradients to isolate EVs from brain tissue. However, the advantages of floatation iodixanol density gradient purification of small EVs have been reported and are further demonstrated here.

Conclusions: Together these findings offer a rigorous technique for purifying whole tissue-derived EVs for downstream analyses, and application of this approach to uncovering molecular changes in AD progression and other neurological conditions.

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Author Contributions

D.G.M, J.M.O, and S.N.H designed the study, interpreted results, and contributed to editing the manuscript. S.N.H, L.S., K.Y.C., and C.R.F. designed and performed experimental procedures.

Conflict of Interest Statement

The authors have no conflict of interest to report.

Keywords

exosomes; microvesicles; neurodegeneration; proteomics; extracellular vesicle

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1. Introduction

Alzheimer's disease (AD) is the leading cause of dementia in older adults, accounting for the majority of the estimated nearly 50 million individuals worldwide living with dementia. Currently, there is no cure for this increasingly prevalent disease, and treatment options harbor minimal efficacy. Additionally, many challenges persist in the discovery of AD biomarkers for disease prediction. A major obstacle in diagnosing and treating AD is the limited understanding of the disease etiology and pathophysiology. Despite the initial discovery of Alzheimer's disease by Alois Alzheimer over a hundred years ago (Alzheimer, 1907), and the increasing prevalence of disease, much remains to be understood about the molecular mechanisms driving AD progression.

Intraneuronal neurofibrillary tangles mainly composed of hyperphosphorylated microtubule-associated tau protein and extracellular neuritic plaques containing amyloid β A4 fibrils are pathognomonic of AD, and likely play roles in the development and progression of neurodegeneration (Alonso et al., 1996; Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b; Hardy and Allsop, 1991; Hardy and Higgins, 1992; Masters et al., 1985). However, neuritic plaques can also be commonly found in the brains of healthy aged individuals, and therefore cannot be used as an isolated diagnostic finding (Baker-Nigh et al., 2015).

Moreover, it is unclear whether plaques formed from insoluble amyloid beta sheets act as a protective sequestering of soluble $A\beta$ 42 oligomers, as these oligomers can cause oxidative stress and tau protein hyperphosphorylation, resulting in damage to synaptic structure and activity (Marques et al., 2003). $A\beta$ peptides can also induce microglial activation, ensuing the release of inflammatory cytokines IL-1, TNF- α , and IFN- γ (Mandrekar et al., 2009). Regardless, there remains dispute as to whether amyloid deposition reliably precedes the remainder of the pathological cascades in the disease progression. It is clear that a better understanding of the pathophysiology of the neurodegeneration that occurs in AD will provide more targeted diagnostic and therapeutic approaches for patients. Furthermore, earlier detection of disease in patients will facilitate more efficacious treatment regimens.

Recent research has revealed that AD-associated pathologic proteins including $A\beta$ and tau are sorted and processed in the endosomal pathway and packaged into EVs for release from cells (Bellingham et al., 2012; Ehehalt et al., 2003; Haass et al., 1992; Rajendran et al., 2006; Saman et al., 2012; Takahashi et al., 2002; Verbeek et al., 2002). More recently, inhibition of EV release results in a reduction in amyloid plaque deposition (Dinkins et al., 2014). In addition, stimulation of EV formation resulted in increased prion protein transmission between cells (Guo et al., 2016). Yet whether EVs may serve a beneficial or harmful role in the progression of disease is unknown (Joshi et al., 2015). Secreted vesicles may serve to trap $A\beta$ and promote clearance by microglia (Yuyama et al., 2012). However, EVs containing neurotoxic tau and amyloid oligomers may also contribute to cell-to-cell propagation of disease when alternative clearance mechanisms are saturated (Agosta et al.,

2014; Joshi et al., 2015; Saman et al., 2012). Further evidence has suggested that altered EV contents in AD-like pathology can induce neuronal death in AD models (Wang et al., 2012). However, the content of these secreted vesicles is unknown. It is likely that investigation into altered brain-derived EV cargo may provide a novel means to further understand the pathogenesis of AD. Furthermore, the utility of EVs as diagnostic markers of disease is an area of rapidly advancing research. Indeed, we have recently demonstrated that EVs largely reflect the state of their progenitor cell (Hurwitz et al., 2016b). Examination of the content of EVs during the progression of AD is therefore an important contribution to earlier detection of disease, and may offer potential therapeutic targets in the future.

In this study, we utilize a common transgenic mouse model designed to recapitulate the pathology of AD: the 5XFAD model, containing five combined familial AD mutations in the amyloid precursor protein (APP) and presenilin (PSEN1) genes. These animals develop accumulating levels of amyloid neuritic plaques corresponding to a decline in cognitive function, progressing rapidly to show signs of detectable plaque load between 2 and 4 months (Oakley et al., 2006). Based on the evidence that small EVs may play a role in Alzheimer's disease development, and may contain altered cargo that lead to the progression of neurodegeneration, we have developed a reproducible technique for isolating and purifying whole brain-derived EVs. Previously, Perez-Gonzalez et al. have described a method to enrich for EVs from brain tissue using a sucrose-density gradient (Perez-Gonzalez et al., 2012). More recently, the Hill lab reported a technique to isolate small EVs using a sucrose-cushion (Vella et al., 2017). However, in the latter study, EVs were extracted from human frontal cortex only rather than whole brain. In addition, the advantages of iodixanol density purification of EVs over stepwise sucrose gradients have been established (Kowal et al., 2016). In the present study, we demonstrate the purity of our vesicle isolates, the advantages of floatation-based density purification, and the utility of this method for downstream proteomic analysis. Furthermore, we compare EV content in healthy age-matched mouse brains to those harboring an AD-like amyloidopathy. This study provides evidence of changes in brain-derived EV content correlating with AD progression, and further demonstrates the potential utility of EVs for early disease detection.

2. Materials and Methods

2.1 Animals

For animal studies, the 5XFAD mouse model of Alzheimer's disease (Oakley et al., 2006), produced using the transgenic stock backcrossed (at least 10 generations) into the C57BL/6J background (MMRRC, 034848-JAX) was utilized. The colony was maintained by mating hemizygous 5XFAD transgenic mice (RRID:IMSR JAX:006554; 5XFAD) to wild-type C57BL/6J mice (RRID:IMSR JAX:000664; B6) to produce 50% hemizygous 5XFAD mice and 50% non-transgenic wild-type controls. Adult female C57BL/6J and adult male 5XFAD mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in the Florida State University College of Medicine animal care facility. All mice for this study were produced by mating hemizygous 5XFAD males to C57BL/6J females. The genotype of progeny was determined via standard PCR for the presenilin 1 (PSEN1) transgene including

internal positive controls for PCR amplification consistent with the instructions provided by the Jackson Laboratory.

Animals were housed individually in a polycarbonate cage (Ancare; 19cm × 29cm × 13cm) with nesting material (Ancare Nestlet), hardwood laboratory bedding chips (Nepco Beta Chip®), and a polycarbonate mouse igloo (Bio-Serv) for enrichment. Mice were maintained under a 12 hour light-dark cycle (7am to 7pm) and given *ad libitum* access to LabDiet® 5001 Rodent Chow and water.

All mice were housed and handled in accordance with Federal animal welfare guidelines and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002) and the Guide for the Care and Use of Laboratory Animals (8th Edition). Experiments were reviewed and approved prior to being carried out by the Institutional Animal Use and Care Committee (IACUC) of the Florida State University (Protocol #1443; Association for Assessment and Accreditation of Laboratory Animal Care International accreditation unit #001031; Office of Laboratory Animal Welfare Assurance #A3854-01).

2.2 Genotyping

At approximately 21 days of age, animals were sedated with an isoflurane inhalation anesthetic (Butler Schein; 029405). Once anesthetized, sterile scissors were used to remove a 2 mm terminal segment of tail. A silver nitrate applicator stick (Butler Schein; 005383) was used to achieve hemostasis. Bupivacaine hydrochloride in sterile isotonic solution (Sigma B5274; 2.5 µg/ml) was applied locally to the excision site for longer-acting pain management. Excision sites were monitored post-surgically for 10 days. Tail biopsies were placed in 250 µL of DirectPCR Tail lysis buffer (Viagen 101-T) with 10 µL Proteinase K solution (Viagen 501-K) for a 6-hour lysis at 55°C. Following lysis, the sample was incubated at 85°C for 45 minutes, then briefly centrifuged. Supernatant containing the genetic material was collected and stored at –20°C for subsequent PCR analysis. Resulting PCR products were separated by gel electrophoresis on to a 1.5% agarose gel (EMD Millipore OmniPur® 2120-OP) for 25 minutes at 110 volts and subsequently imaged on a Bio-Rad Gel Doc™ XR.

2.3 Animal perfusion

To collect brain tissue for immunohistochemistry, animals were injected intraperitoneally with ketamine (Butler Schein; 100 mg/kg) and xylazine (Vedco; 10 mg/kg) as previously described (O’Neal-Moffitt et al., 2015). Animals were then fixed by intracardial perfusion of 0.9% sterile saline, followed by 4% paraformaldehyde (PFA). Whole brains were removed and placed in 30% sucrose at 4°C, then frozen at –20°C until further processing. To obtain coronal tissue slices, brains were covered in Tissue-Tek embedding compound (VWR 25608–930) and sectioned by cryotome into 40 µm thick slices. Slices were placed on slides and stored at –80° C until processed by immunohistochemistry.

2.4 Immunohistochemistry

Brain sections were post-fixed in 4% PFA (pH 7.4) for 10 minutes before amyloid plaque immunohistochemistry staining, as described in detail by O'Neal-Moffitt and colleagues (O'Neal-Moffitt et al., 2015). Sections were imaged using an EVOS FL Imaging System (Thermo, AMF4300). Images were processed with CorelDraw X5. Representative images from 3–4 animals at each age are shown.

2.5 Isolation of EVs from whole brains

Whole brains were extracted from WT and 5XFAD mice at two and six months of age following rapid decapitation, and were frozen at -80°C until further processing. The two month mice included three WT (two male and one female) and three 5XFAD animals (two male and one female). The six month brains were harvested from three WT (one male and two female) and three 5XFAD (two male and one female) animals. To homogenize the tissue, 10 mL of activating solution in Hibernate E medium [1 mg/mL papain; 5.5 mM L-cysteine; 1.1 mM EDTA; 0.067 mM 2-mercaptoethanol] was added to each brain, according to Perez-Gonzalez (Perez-Gonzalez et al., 2012). Brains in solution were incubated at 37°C for 20 minutes before gentle disassociation (30 strokes) with a loose-fit Dounce homogenizer (DWK Life Sciences, 885300–0015, pestle clearance 0.889–0.165 mm). Following homogenization, a differential centrifugation strategy was employed. Tissue fragments and cells were pelleted and discarded by centrifugation at 500 g for 5 minutes, then further centrifuged at 2,000 g for 10 minutes to pellet large cellular debris. Supernatants were centrifuged at 10,000 g for 30 minutes to pellet and discard unwanted large vesicles. Samples were then filtered through a $0.45\text{ }\mu\text{m}$ filter, and ultracentrifuged at 100,000 g for 2 hours in an SW41 Ti rotor to concentrate small EVs. EV pellets were resuspended in 1.5 mL of 0.25 M sucrose buffer [10 mM Tris, pH 7.4] for gradient purification.

Top-loaded sedimentation gradients were performed as previously described in detail (Hurwitz et al., 2016a). Bottom-loaded floatation gradients were adapted from Kowal et al. (Kowal et al., 2016) and prepared as described (Hurwitz et al., 2017b). Briefly, 60% iodixanol (Optiprep; Sigma, D1556) was added 1:1 to EV/buffer solution and transferred to the bottom of a 5.5 mL ultracentrifugation tube. Subsequently, 1.3 mL of 20% iodixanol and 1.2 mL of 10% iodixanol were layered on top. Gradients were ultracentrifuged at maximum speed in an MLS-50 rotor (268,000 g) for 50 minutes. Ten fractions of 490 μL were taken from the top of the gradient, diluted in PBS, and pelleted again for 2 hours at 100,000 g in an SW41 Ti rotor to rid samples of iodixanol. Purified fraction pellets were lysed in 40 μL of strong lysis buffer [5% SDS; 10 mM EDTA; 120 mM Tris-HCl pH 6.8; 2.5% 2-mercaptoethanol; 8 M urea] with the addition of HALT protease inhibitor (Thermo, 78438).

2.6 Immunoblot analysis

To prepare brain homogenates, tissue was removed from the frontal lobe, lysed in strong lysis buffer (detailed above) with proteinase inhibitor, homogenized by pipette, and centrifuged at $10,000 \times g$ for 10 minutes to discard whole cells and intact cellular debris. For confirmation of EV proteins in purified lysates, 5X Laemmli sample buffer [10% SDS, 250 mM Tris pH 6.8, 1 mg/mL bromophenol blue, 0.5 M DTT, 50% glycerol, 5% BME] was added to EV isolates for a final concentration of 1X. For immunoblot analysis of CD63,

DTT and BME were omitted from lysis and sample buffers to allow the protein to run in non-reducing conditions. Gel electrophoresis and western blotting was performed as previously described (Hurwitz et al., 2017b). Equal volume of gradient lysates were loaded into an SDS-PAGE gel. Blots were probed with the following antibodies: Alix (Q-19; Santa Cruz Biotechnology), HSC70 (B-6; Santa Cruz), TSG101 (C-2; Santa Cruz), CD63 (TS63; Abcam), Rab8a (63-BJ ; Santa Cruz), and CD81 (H-121 ;Santa Cruz), rabbit anti-mouse IgG (Genetex, 26728), rabbit anti-goat IgG (Genetex, 26741), goat anti-rabbit IgG (Fab fragment) (Genetex, 27171). Blots were imaged using an Image Quant LAS4000 (General Electric) and processed with ImageQuant TL v8.1.0.0 software, Adobe Photoshop CS6 and CorelDraw Graphic Suite X5.

2.7 Transmission electron microscopy

Gradient-purified EVs were re-suspended in particle-free PBS and prepared on electron microscope grids as described (Hurwitz et al., 2017b; Lässer et al., 2012). Images were taken on an FEI CM120 TEM instrument.

2.8 SDS-PAGE and in-gel digestion

Vesicle protein in gradient fractions containing EVs was quantified using the fluorescence-based EZQ™ Kit (Thermo, R33200). Equal mass (10 µg) of EV protein was run 0.5 inches into a 15% polyacrylamide gel (Lonza 59510) for gel purification, then fixed and Coomassie-stained, as previously detailed (Meckes, 2014). Lanes were cut into 1 mm³ cubes for trypsin digestion, as described by Rider et al. (Rider et al., 2016).

2.9 Mass spectrometry

An externally calibrated Thermo Q Exactive HF (high-resolution electrospray tandem mass spectrometer) was used in conjunction with Dionex UltiMate3000 RSLCnano System. A 5 µL sample was aspirated into a 50 µL loop and loaded onto the trap column (Thermo µ-Precolumn 5 mm, with nanoViper tubing 30 µm i.d. × 10 cm). The flow rate was set to 300 nL/min for separation on the analytical column (Acclaim pepmap RSLC 75 µM × 15 cm nanoviper). Mobile phase A was composed of 99.9 H₂O (EMD Omni Solvent), and 0.1% formic acid and mobile phase B was composed of 99.9% ACN, and 0.1% formic acid. A 60 minute linear gradient from 3% to 45% B was performed. The LC eluent was directly nanosprayed into Q Exactive HF mass spectrometer (Thermo Scientific). During the chromatographic separation, the Q Exactive HF was operated in a data-dependent mode and under direct control of the Thermo Excalibur 3.1.66 (Thermo Scientific). The MS data were acquired using the following parameters: 20 data-dependent collisional-induced-dissociation (CID) MS/MS scans per full scan (350 to 1700 m/z) at 60000 resolution. MS2 were acquired in centroid mode at 15000 resolution. Ions with single charge or charges more than 7 as well as unassigned charge were excluded. A 15 second dynamic exclusion window was used. All measurements were performed at room temperature. Resultant raw files were searched with Proteome Discoverer 2.0 using MS-Amanda, SequestHT, and Mascot as the search engines using modified mouse FASTA database and percolator as peptide validator. SequestHT and Mascot search parameters used were as follows: enzyme name = Trypsin, maximum missed cleavage = 2, minimum peptide length = 6, maximum peptide length = 144, maximum delta Cn = 0.05, precursor mass tolerance = 10 ppm, fragment mass tolerance = 0.2 Da, variable

modifications: oxidation/+16 Da (M) and acetyl/+42 Da (N), and dynamic modifications: carbamidomethyl/+ 57.021 Da (C). Protein and peptide identities were validated using Scaffold (version 4.7.1, Proteome Software Inc., Portland, OR, USA) software. Peptide identity was accepted if Scaffold Local FDR algorithm demonstrated a probability greater than 95.0%. Likewise, protein identity was accepted if the probability level was greater than 99.0% and contained a minimum of two recognized peptides.

2.10 Enrichment analysis

Cellular compartment enrichment of proteins identified by mass spectrometry in this study was analyzed using FunRich v3 (Benito-Martin and Peinado, 2015; Pathan et al., 2015). Compartments with the highest enrichment are shown in each dataset. Biological process (GOTERM_BP_DIRECT) enrichment of sedimentation and floatation-isolated EV proteins in Figures 2 and 3 were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (Huang et al., 2009a, b). Fold enrichment of protein localization in Figure 4A and biological process enrichment changes between AD and WT animals were performed using FunRich v3. Overlap of proteins identified with the known Vesiclepedia database of EV proteins were analyzed in FunRich v3 using either the mouse (Figure 5C and 6C) or human (Supplementary Figure 3) UniProt database.

2.11 Statistical analysis

Statistical significance of enrichment categories was determined by FunRich v3 and DAVID v6.8. Only significant categories ($p < 0.05$) are displayed in figures. Significant differences in total EV protein harvested from WT and AD brains in Figure 5A were determined by a two-way analysis of variance (ANOVA) with a post-hoc Tukey HSD test.

3. Results

3.1 Suboptimal density gradient purification of whole brain-derived EVs presents challenges in biologically meaningful isolation techniques.

Historically, transgenic mouse models harboring mutant APP and PSEN1 proteins have been utilized for biomedical Alzheimer's research. These models are aimed to recapitulate the overproduction and accumulation of amyloid beta seen in many AD patients, and are focused on amyloid protein as both the chief pathogenic factor and major biomarker of disease progression. In our hands, 5XFAD mice characteristically developed rapid plaque deposition in the hippocampus (Figure 1). Amyloid plaques were observed as early as 4 months of age in predominately the CA1 region of the hippocampus, an area which has previously been described as particularly vulnerable to early uptake and sequestration of A β 42 (Bahr et al., 1998). Plaque burden throughout the rest of the hippocampus and cortex continued to increase with age in AD animals.

Although plaques reliably accumulate in several models of AD, amyloid beta remains a controversial and inconsistent biomarker of disease. Given the recent interest in EVs as markers of disease progression, we aimed to investigate the utility of vesicle protein content in reflecting AD pathology. To closely examine the cargo of brain-derived EVs, we purified whole mouse brain-derived EVs by tissue homogenization as described by Perez-Gonzalez

et al. (Perez-Gonzalez et al., 2012), followed by differential centrifugation, and subsequent purification by sedimentation on a top-loaded stepwise iodixanol density gradient. Immunoblot analysis of the 12 gradient fractions demonstrated vesicle protein predominately in fractions 6 and 7, corresponding to densities of 1.07 to 1.09 g/mL (Figure 2A and Supplementary Figure 1). Notably, we have recently showed Rab8a to be a universal EV marker (Hurwitz et al., 2016b), which was present in minimal levels in sedimentation-isolated brain-derived EVs. We have previously shown fraction 6 vesicles to be highly enriched in small EVs (Hurwitz et al., 2017a; Hurwitz et al., 2016a; Hurwitz and Meckes, 2017). Protein in fraction 6 was quantified and in-gel digested for mass spectrometry analysis. A total of 865 proteins were identified in brain-derived EVs (Supplementary Table 1), and were enriched in known exosomal, cytoplasmic, and membrane-associated proteins (Figure 2B). Many identified proteins were involved in transport, adhesion, and vesicular processes within the cell (Figure 2C). Interestingly, EV proteins were also involved in aging, protein folding, and brain development, suggesting that vesicle cargo may represent important cellular processes reflective of disease state. However, despite the enrichment in EV proteins following iodixanol gradient purification, electron microscopy of vesicles revealed the presence of cellular debris among small EVs (Figure 2D), likely due to co-purified tissue fragments or other lipid particles following gradient separation.

3.2 Optimized floatation density gradient provides increased purity of EVs from whole brains.

Floatation-based density gradients often provide increased separation and purification of low density subcellular compartments. In efforts to increase the purity of our brain-derived EV isolates, we resuspended crude EV pellets on the bottom of a stepwise iodixanol density gradient for floatation-based separation, adopted from Kowal et al. (Kowal et al., 2016) (Figure 3A). Immunoblot analysis determined the vast majority of vesicle protein floated to fraction 2 of the gradient, with the exception of a non-specific EV marker, HSC70 (Figure 3B and Supplementary Figure 2). In this technique, Rab8a was also present more abundantly. Interestingly, we did not observe vesicle populations in the denser fraction of the gradient, as previously noted in cell culture (Hurwitz et al., 2017b; Kowal et al., 2016). It is possible that the release of denser EV populations is specific for certain cell types, or may even be an *in vitro* phenomenon. Vesicles in fraction 2 were examined by electron microscopy, revealing highly pure and abundant, clear membrane-bound vesicles between 50 and 200 nm in size (Figure 3C). EV protein in this fraction was lysed and prepared for mass spectrometry analysis. A total of 1,045 proteins were identified in brain-derived EVs (Supplementary Table 2), and were again enriched in biological processes including transport, metabolic activities, vesicle trafficking, and nervous system development (Figure 3D).

Direct comparison of EV proteins identified using sedimentation- versus floatation-based isolation techniques was performed. Proteins localized to endomembrane compartments, MVBs, postsynaptic membranes, and lamellipodia were more highly enriched in EV isolates using the floatation gradient (Figure 4A). MVBs have previously been demonstrated to traffic along the axon to the synaptic terminal before membrane fusion and exosome release (Janas et al., 2016), and small EVs have been shown to “surf” on filopodia before

endocytosis (Heusermann et al., 2016). In contrast, sedimentation-isolated samples contained a higher proportion of proteins present in other organelle membranes, extracellular chylomicrons, and lipoprotein particles. These findings suggest the sedimentation-isolated EVs may be contaminated with membrane fragments or other extracellular lipid particles that co-migrate with EVs in density fractions. In addition, relative abundance of well-known EV markers previously described (Kowal et al., 2016; Vella et al., 2017) was compared between isolation techniques (Figure 4B). EV proteins including tetraspanin CD81, ESCRT-associated protein Alix, Rab10, Flotillin proteins, and charged multivesicular body protein 4B (CHMP4B) were found in higher abundance in mass spectrometry analysis of equal protein mass in floatation gradient samples compared to sedimentation gradient fractions. Conversely, endoplasmic reticulum non-EV proteins calnexin and ATP2A2 were found in relatively greater abundance in sedimentation fractions. These findings were similarly confirmed by immunoblot (Figure 4C). While both floatation- and sedimentation-isolated EV samples were depleted of Calnexin compared to starting brain material, floatation purification demonstrated increased elimination of the contaminating ER protein that is also a component of apoptotic bodies. In contrast, EV protein CD81 was more highly enriched in floatation-isolated EVs compared to sedimentation fractions or brain homogenate. Furthermore, while non-specific EV protein HSC70 was present in EV fractions from both methods (Figures 2A and 3B), the protein was enriched in the sedimentation fraction compared to cell lysates, but not in the floatation sample (Figure 4C). As HSC70 has been shown to associate with clathrin in clathrin-coated pits (Jiang et al., 2000), this finding suggests a greater abundance of non-EV protein to be present in the sedimentation fractions. Altogether, the immunoblot analysis, EM, and comparative LC-MS/MS protein enrichment demonstrate that the density floatation separation protocol offers an improved method to purify whole brain-derived EVs.

3.3 Comparison of control and AD brain EVs identifies potential biomarkers of disease progression.

As accumulating evidence has proposed that EV cargo can reflect the status of cellular activity and health, we hypothesized that brain-derived EVs may harbor proteins that reflect the progression of AD. Interestingly, AD brains appeared to contain greater amounts of EV protein than age-matched WT brains at several different ages (Figure 5A). These data suggest likely differences in vesicular content during disease state. Importantly, no difference was seen in the enrichment of proteins involved in apoptotic processes in AD brain-derived EVs (data not shown). To further explore changes in EV content, brain-derived EVs from 6-month old healthy and AD animals were purified by the floatation gradient method (Supplementary Table 3), as amyloid plaques were observed abundantly at this age in the hippocampus (Figure 1). Proteins from all brains were enriched in extracellular vesicle, cytoplasmic, and membrane compartments (Figure 5B). To ensure reproducibility of the isolation technique, the overlap of EV proteins identified in each biological replicate of WT and AD brains was compared (Figure 5C). The vast majority of identified proteins remained consistent between replicates, demonstrating the consistency of EV protein isolation and the proteomics workflow. Of note, the WT samples contained an additional female brain, while the AD samples contained an additional male brain. Regardless, it appeared that EV protein harvested remained relatively similar amongst brain samples

(Figure 5C). Comparison to the Vesiclepedia database of known mouse EV proteins revealed many proteins not previously identified in mouse EVs (Figure 5D). However, of significance, the number of previously identified mouse EV proteins pales in comparison to the human EV database. Indeed, comparison of the brain EV proteins identified in this study to the human EV database revealed an additional 50% of proteins to overlap with the Vesiclepedia bank (Supplementary Figure 3). Despite the improved method of EV isolation described here, it still remains possible that some of these proteins could represent contaminating non-EV proteins. However, based on the purity of isolates seen in Figure 3, it is likely that many proteins in our dataset represent uncharacterized mouse-derived vesicular proteins, as well as brain parenchymal-derived EVs that will contribute to the growing compendium of EV cargo for other researchers.

Further comparison of proteins increased or decreased by over two-fold change between WT and AD 6-month brain-derived vesicles revealed the potential of EVs to reflect AD pathological processes (Figure 5E). Strikingly, proteins decreased in vesicles harvested from AD brains were involved in brain development, aging, immune response, and oxidation-reduction maintenance. These proteins may reflect essential molecular processes that are disrupted in neurodegenerative diseases such as AD. Interestingly, changes in abundance of proteins involved in drug response and transport, including many solute and ion transporters, may represent disturbances in both cellular homeostasis of membrane potential as well as blood brain barrier integrity. For instance, multidrug resistance protein (MDR1A) was decreased in EVs from AD brains, supporting potential interruptions to normal brain clearance mechanisms that have been proposed in AD (Deane and Zlokovic, 2007; Sagare et al., 2013; Zlokovic et al., 2000) (Table 1). Surprisingly, proteins capable of cleavage and misfolding, including amyloid-beta A4 protein (APP) and synucleins were also found to be decreased in AD brain EVs. This observation supports disruption to the brain clearance of proteins with a propensity to aggregate in the 5XFAD model, and may explain the rapid accumulation of plaques in these mice. Respective decreases in dynamin-recruiter amphiphysin and increases in transferrin receptor protein in AD EVs suggest possible defects in global brain endocytosis, which may also in part explain the elevated protein content in EVs from AD brains (Figure 5A). Altogether, the identification of mouse brain vesicular protein changes in the context of neurodegeneration, as well as a rigorous method for purification of parenchymal EVs, will drive future focused research avenues in AD pathophysiology.

3.4 Young animal brain EV proteomes show early molecular changes in neurodegeneration.

To investigate the cargo of EVs that may reflect the progression of early to later-stage AD, vesicles were purified from 2-month old control and 5XFAD mouse brains (Supplementary Table 4). Again, protein cargo between biological replicates remained highly consistent (Figure 6A). Proteins identified by mass spectrometry in WT and AD brain EVs were compared to the mouse and human Vesiclepedia databases, and showed similar overlap as before (Figure 6B and Supplementary Figure 3). Interestingly, many of the biological processes altered in 6-month old animal EVs did not appear as disrupted in younger animals (Figure 6C), whereas sorting of many vesicle proteins was found to be altered in AD animals

regardless of age, suggesting early differences in EV cargo that precede amyloid buildup (Figure 6D). These proteins may represent potential improvements for early detection of neurodegenerative disease. On the other hand, a number of identified proteins were only altered in older animal brain EVs. Strikingly, protein levels of transmembrane protein 33 (Tmem33), an endoplasmic reticulum protein responsive to misfolded proteins, and delta-catenin, a cadherin-binding protein at the synapse and in adherens junctions, were greatly increased in EVs from only older AD brains (Table 1). Delta-catenin has been implicated in maintaining synaptic structure and may interact with the Alzheimer's protein presenilin (Jun et al., 2012; Kosik et al., 2005; Zhou et al., 1997), while Tmem33 has been characterized as a binding partner of reticulon proteins that interact with β -secretase (He et al., 2004; Urade et al., 2014). These likely represent proteins involved in AD progression, and additionally warrant future investigation as biomarkers of neurodegeneration.

4. Discussion

Recent evidence has suggested that brain-derived EVs may harbor molecular information reflective of neurodegeneration and may further contribute to the pathogenesis of disease (Cheng et al., 2017; Janas et al., 2016; Lim and Lee, 2017; Trotta et al., 2018). Research into the content and function of extracellular vesicles in brain disease has rapidly expanded over the past several years, however isolation and purification of secreted vesicles remains a challenge. In this study we demonstrate the optimization of a protocol to efficiently purify EVs from whole brains, and show the limitations of vesicle purification from complex tissues on a sedimentation gradient. Previously the Levy and Hill labs have developed novel methods to purify brain EVs by sucrose density isolation (Perez-Gonzalez et al., 2012; Vella et al., 2017). Here we offer an alternative method based on the latest evidence demonstrating the superiority of iodixanol density floatation gradient EV purification for protein characterization (Kowal et al., 2016). We further uniquely provide confirmation of the utility of this method in downstream mass spectrometry analysis. This protocol could also be easily adapted to purify and characterize EVs secreted into the extracellular space of other complex tissues such as tumors.

In this study, we demonstrate the application of this method to characterizing global EV content from healthy and 5XFAD mouse brains. Currently, the mouse database of EV proteins contains only 17% of the quantity of human EV proteins discovered. Through this analysis, we contribute substantially to the current database of known mouse EV proteins, which will benefit other EV researchers. We also highlight vesicle protein cargo altered in the context of neurodegeneration, and show evidence of early and later changes. While amyloid plaque accumulation, neuronal death, and neuroinflammation in the 5XFAD mouse model progresses much more rapidly than in humans, previous evidence has indicated that many molecular alterations in these mice reflect human pathology (Maarouf et al., 2013).

Our efforts to optimize a reproducible method for downstream proteomic analysis of brain EVs may offer novel insight into undescribed molecular changes in the 5XFAD model to help understand the progression of disease in these animals. Based on accumulating evidence suggesting the role of EVs in contributing to neurodegeneration seen in AD, it is likely that differences in EV content may be important in propagation of processes such as

neuroinflammation. It is worth noting that one limitation of this method is that a portion of EVs isolated may actually represent vesicles in the CSF or brain circulation, adding complexity to completely understanding content changes or clearance events. If exclusion of all plasma vesicles is desired, animals should be perfused with saline before brains are collected. Further investigation into biological alterations to EV uptake by neuronal and glial cells, and clearance into CSF and plasma in AD may help in understanding the differences seen in brain-derived versus circulating plasma EVs.

5. Conclusion

Overall, these findings reveal new insights into dysregulation of brain cell functions in the context of neurodegeneration, the utility of differences in EV content to investigate mechanisms of AD pathogenesis, a refined method to isolate whole brain-derived EVs, and the potential application of this technique for the discovery of early biomarkers of disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Mia Hartley for assistance with animal care and immunohistochemistry. We thank Richard Nowakowski for providing the 5XFAD mice and the FSU Laboratory Animal Resources for assistance in caring for the animals used in this study. We also thank Xia Liu, Rakesh Singh, and the Florida State University Translational Science Laboratory for help with the mass spectrometry analyses, and the Florida State University Biological Science Imaging Resource facility for assistance with the electron microscopy. This study was supported by a grant from the Florida Department of Health Ed and Ethel Moore Alzheimer's Disease Research Program awarded to D.G.M. (6AZ11).

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Highlights:

1. Validation of an optimized iodixanol density gradient protocol to efficiently and rigorously purify EVs from whole mouse brains.
2. Demonstration of the limitations of vesicle purification from complex tissues on a sedimentation density gradient.
3. Application of this method to characterizing and comparing global EV content from healthy and 5XFAD mouse brains.

Figure 1. Hippocampal amyloid plaque deposition increases with age in the 5XFAD mouse model.

Longitudinal comparison of hippocampal amyloid plaque deposition in the 5XFAD model by immunohistochemistry. (mo), month.

Figure 2. Suboptimal density gradient purification of whole brain-derived extracellular vesicles for mass spectrometry analysis.

A) Small extracellular vesicles were enriched from whole mouse brains (n=6) using a top loaded sedimentation density gradient isolation protocol. Immunoblot analysis revealed diffuse presence of EV markers across the gradient. EV protein in fraction 6 was analyzed by mass spectrometry. Enrichment of B) cellular compartment localization and C) biological processes of proteins identified in brain-derived EVs. D) Electron microscopy revealed the presence of small vesicles (arrowheads) among other cellular debris.

Figure 3. Flootation density gradient enrichment of brain-derived vesicles yields highly pure EV protein.

A) EVs were enriched from mouse brains (n=6) using a bottom-loaded iodixanol density flotation gradient protocol. B) Immunoblot analysis revealed EVs to be consistently isolated in fraction 2 of the gradient. C) Electron microscopy of fraction 2 vesicles demonstrates highly pure, membrane-bound 50–200 nm vesicles. D) Enrichment of biological processes of proteins identified by mass spectrometry analysis.

Figure 4. Flootation-based separation produces a higher quality EV enrichment than a sedimentation-based approach.

A) FunRich analysis of cellular compartment enrichment in EV proteins identified using floatation- versus sedimentation-based isolation approaches. B) Relative spectral count abundance of common EV proteins and non-EV proteins in floatation- and sedimentation-isolated brain EV samples. C) Immunoblot analysis demonstrating enrichment of EV protein CD81 and depletion of Calnexin and HSC70 in floatation-isolated EVs. BH, brain homogenate. Float, floatation. Sed, sedimentation.

Figure 5. Comparison of brain-derived EVs from 6-month old WT and 5XFAD mice reveals differences in protein cargo.

A) Total protein (µg) harvested per mouse brain across several ages was measured by a sensitive fluorescent based protein quantification assay. Bars represent average protein mass per brain. B) Enrichment of cellular compartment of proteins following mass spectrometry of brain EVs from 6-month old animals. C) Overlap of unique proteins identified by mass spectrometry in 6 month WT and 5XFAD biological replicates. D) Overlap of EV proteins identified in 6 month WT and disease brains with previously identified mouse EV proteins in the Vesiclepedia database. E) Comparison of biological process enrichment between WT and 5XFAD brain EV protein. Proteins increased or decreased by 2-fold or greater compared to the WT dataset were included in the analysis. *, $p < 0.05$; **, $p < 0.01$.

Figure 6. Analysis of brain EVs from young mice compared to aged animals provides evidence of progressive neurodegenerative pathology.

A) Overlap of unique proteins identified by mass spectrometry in 2 month WT and 5XFAD biological replicates. B) Comparison of EV proteins found in 2-month WT and 5XFAD brains with the Vesiclepedia database of known mouse vesicle proteins. C) Comparison of biological processes enriched in EV proteins from 2 month WT and 5XFAD brains. Proteins increased or decreased by 2-fold or greater compared to the WT dataset were included in the analysis. D) Overlap in unique vesicle proteins increased or decreased by 2-fold or greater in WT and 5XFAD brains from 2- and 6-month old animals.

Table 1.

Relative EV protein abundance identified in WT and 5XFAD mouse brains.

Protein name	Gene symbol	Fold change in 6 mo	Fold change in 2 mo
Transmembrane protein 33	Tmem33	INF	N/A
Catenin delta-2	Ctnnd2	36	N/A
26S proteasome non-ATPase regulatory subunit 3	Psmd3	10	6.3
Cytochrome b-c1 complex subunit 1, mitochondrial	Uqcrc1	7.5	N/A
Lipid phosphate phosphatase-related protein type 4	Lppr4	7	2.5
Carnitine O-palmitoyltransferase 1, brain isoform	Cpt1c	5.7	1.3
Transferrin receptor protein 1	Tfrc	3.2	1.6
Immunoglobulin superfamily member 21	Igsf21	2.7	1.8
Heat shock 70 kDa protein 1-like	Hspa11	2.3	N/A
Brain acid soluble protein 1	Basp1	0.5	1
Neuromodulin	Gap43	0.5	1.1
Amphiphysin	Amph	0.4	1.6
Microtubule-associated protein 2	Map2	0.4	1.5
Neurocalcin-delta	Ncald	0.4	1
Acetyl-CoA acetyltransferase, cytosolic	Acat2	0.4	0.9
Amyloid beta A4 protein	App	0.3	0.1
Alpha-synuclein	Snca	0.3	1.2
Beta-synuclein	Sncb	0.3	1.2
Microtubule-associated protein 4	Map4	0.2	0.8
Multidrug resistance protein 1A	Abcb1a	0.2	2.3
Glia maturation factor beta	Gmfb	0.08	4.2
Oxidation resistance protein 1	Oxr1	0.08	1.8
Ubiquitin-conjugating enzyme E2 L3	Ube2l3	0.07	1.2
Superoxide dismutase [Mn], mitochondrial	Sod2	0	1.1

* Fold change = greater than 2-fold change in spectral count abundance, baseline WT. mo, months.

Red = increased by greater than 2-fold in AD brain EVs

Blue = decreased by greater than 2-fold in AD brain EVs.