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Altered proteomics in brain extracellular vesicles from depressed individuals who died by suicide implicates synaptic processes

Pascal Ibrahim PhD[1,2], Haruka Mitsuhashi BSc[1,2], Lorne Taylor[3], Jenna Cleyle MSc[3], Naguib Mechawar PhD[1,2,4], Corina Nagy PhD[1,2,4,#], Gustavo Turecki MD, PhD[1,2,4,*,#],

[1]Integrated Program in Neuroscience, McGill University, Montreal, Quebec, Canada[2]McGill Group for Suicide Studies, Douglas Mental Health University Institute, Verdun, Quebec, Canada[3]Proteomics and Molecular Analysis Platform, Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada[4]Department of Psychiatry, McGill University, Montreal, Quebec, Canada.

[*]Corresponding author: Gustavo Turecki, MD, PhD, Department of Psychiatry, McGill University, 6875 Boulevard LaSalle, Montréal, QC, CA, Office: F-3125, Frank B. Common Pavilion, Canada. (gustavo.turecki@mcgill.ca)
[#]These authors share senior authorship.

Abstract

Background: Major depressive disorder (MDD) is a common and debilitating disorder whose molecular neurobiology remains unclear. Extracellular vesicles (EVs) are small vesicles that are released by cells and are involved in intercellular communication. They carry bioactive molecules, such as proteins, that reflect the state of their cell of origin. In this study, we sought to investigate the proteomic cargo of brain EVs from depressed individuals as compared to EVs from matched neurotypical individuals. In addition, we investigated how the EV proteomic cargo compares to the proteomic profile of bulk tissue.

Methods: Using mass spectrometry and label-free quantification, we investigated the EV and bulk tissue protein profile from anterior cingulate cortex samples from 86 individuals. We performed differential expression analysis to compare cases and controls, followed by in silico analysis to determine potential implicated functions of dysregulated proteins.

Results: Extracellular vesicles display distinct proteomic profiles compared to bulk tissue. Differential expression analysis showed that 70 proteins were differentially packaged in EVs in MDD, while there was no significant difference in protein levels between groups in bulk tissue. In silico analysis points to a strong role of these differential EV proteins in synaptic functions.

Conclusion: To our knowledge, this is the first study to profile EV proteins in depression, providing novel information to better understand the pathophysiology of MDD. This work paves the way for discovering new therapeutic targets for MDD and prompts more investigations into EVs in MDD and other psychiatric disorders.

Keywords: extracellular vesicles, proteomics, LC-MS/MS, depression, postmortem

Significance Statement

Major Depressive Disorder (MDD) is a debilitating disorder with a global impact, and unfortunately, current treatments are not effective in all patients. There is an urgent need to develop novel treatments for MDD, which requires a deeper understanding of the molecular mechanisms underlying the disorder. In this study, we investigated the protein cargo in extracellular vesicles (EVs) in postmortem human brain. We found that EVs exhibit a proteomic profile that differs from the tissue of origin, emphasizing the importance of studying brain EVs, as they provide information that is otherwise overlooked. We also compared the EV proteomic profiles between controls and individuals who had MDD. We found a significant difference in the levels of 70 proteins, and that these proteins have implications in synaptic function. This study adds to our knowledge around the molecular underpinnings of MDD, which could potentially bring us closer to developing new treatments.

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INTRODUCTION

Major depressive disorder (MDD) is a common and debilitating disorder, which is currently a leading contributor to the global disease burden today.[1,2] Despite progress on our understanding of the pathophysiology of MDD,[3] the underlying mechanisms remain poorly understood. Investigating brain proteomic alterations associated with MDD can potentially help fill this gap.

Extracellular vesicles (EVs) are lipid-bilayered particles that are released by most cell types, including those of the central nervous system (CNS).[4] Extracellular vesicles exist in a wide range of sizes, but for the purpose of our study, we use the term "EVs" to refer to vesicles that range from 30 to 200 nm in diameter. Extracellular vesicles carry bioactive proteins, which reflect the state of the cell of origin and may elicit a change in function if taken up by a recipient cell,[5,6] making EVs interesting targets to study molecular changes associated with the pathophysiology of CNS disorders.[7,8]

To date, there have only been a few studies that profiled the proteomic cargo of EVs directly investigating brain tissue. These studies focused on Alzheimer's[9-12] and Amyotrophic lateral sclerosis.[13] To our knowledge, studies have yet to investigate EV proteomic changes in psychiatric disorders. Therefore, here we sought to profile the brain proteomic EV cargo associated with MDD, focusing on the anterior cingulate cortex (ACC), a brain region which has consistently been implicated in depression.[14-16] We compared the EV proteomic cargo to the proteomic profile of the corresponding bulk tissue and showed that they are different. Subsequently, we compared protein levels between MDD and controls in EVs and bulk tissue, and although we found no significant difference of protein levels in bulk tissue, we found 70 proteins differentially packaged in EVs between cases and controls. In silico functional follow-up was then performed, and we found that most of these proteins were associated with synaptic processes.

MATERIALS AND METHODS

Human Brain Samples

Frozen samples from the ACC (Brodmann area 24) were obtained from the Douglas-Bell Canada Brain Bank. Tissue dissections were performed as previously described.[17] This study included 86 subjects, 43 (23 male [M]/20 female [F]) of which were neurotypical controls who died suddenly without a prolonged agonal state (control [CTRL] group). The other 43 subjects (23M/20F) died by suicide in the context of a major depressive episode (MDD group) (Table S1). Psychological autopsies were performed by trained clinicians using proxy-based structured diagnostic interviews[18] with informants best acquainted with the deceased, and Diagnostic and Statistical Manual for Mental Disorders 5 criteria were used to elicit diagnoses. The Research Ethics Board of the Douglas Mental Health University Institute provided ethical approval for this study, and the families of the deceased provided written informed consent.

EV Isolation

Extracellular vesicles were isolated using the size exclusion chromatography method developed in our previous study.[19] Briefly, frozen brain tissue was incubated in Hibernate E medium (Thermo Fisher Scientific) with collagenase III (Worthington) for 20 min at 37 °C and then was subjected to 3 rounds of centrifugation: 300 ×*g* for 5 min, 2000 ×*g* for 10 min, and 10 000 ×*g* for 30 min. The supernatant of the last spin was then overlaid on a size exclusion column (Izon Science) where fractions were collected according to the manufacturer's protocol and concentrated using Amicon centrifugal filters (Millipore-Sigma).

Protein Extraction

A total of 10-20 mg of ACC tissue from each of a subsample of 20 subjects (Table S2) was used to extract proteins. Briefly, tissue was placed in 100 µL 1X radioimmunoprecipitation assay (RIPA) buffer and homogenized on ice. The samples were left on ice for 15 min and then centrifuged at maximum speed at 4 °C for 10 min. The supernatant was then collected, and proteins were quantified using Pierce bicinchoninic acid (BCA) Protein Assay (Thermo Fisher Scientific).

Ultra-High-Performance Liquid Chromatography and Tandem Mass Spectrometry

Proteomic analysis was performed on equivalent amounts of EVs from each subject, as well as on proteins extracted from bulk ACC tissue, at the McGill University Health Centre Research Institute. Nanodrop quantification was performed in triplicates for absorbance at 280 nm (protein concentrations) to quantify samples prior to sending to the service provider. A solution of approximately 3.125 µg/µL was then prepared for each subject and used for analysis. For each sample, lysates were loaded onto a single stacking gel band to remove lipids, detergents, and salts. The single gel band containing all proteins was reduced with dithiothreitol, alkylated with iodoacetic acid, and digested with trypsin. Extracted peptides (2 µg) were re-solubilized in 0.1% aqueous formic acid and loaded onto an Acclaim Pepmap precolumn (75 µm ID × 2 cm with 3 µm C18 beads) (Thermo Fisher Scientific) and then onto an Acclaim Pepmap EASY-Spray analytical column (75 µm × 50 cm with 2 µm C18 beads) (Thermo Fisher Scientific). Separation was performed using a Dionex Ultimate 3000 uHPLC (Thermo Fisher Scientific) at 250 nL/min with a gradient of 2%-35% organic (0.1% formic acid in acetonitrile) over 3 hr. Peptides were analyzed using an Orbitrap Fusion Mass Spectrometer (Thermo Fisher Scientific) operating at 120 000 resolution (full width at half maximum (FWHM) in MS1) with higher-energy collisional dissociation (HCD) sequencing (15 000 resolution) at top speed for all peptides with a charge of 2^+ or greater.

Label-Free Quantification

For label-free quantification (LFQ), raw MS files were processed using the MaxQuant software (ver. 2.4.2.0, Max-Planck Institute of Biochemistry, Department of Proteomics and Signal Transduction) by which the precursor MS signal intensities were determined, and HCD-tandom mass spectrometry (MS/MS) spectra were de-isotoped and filtered such that only the 10 most abundant fragments per 100-m/z range were retained. Peptides were identified by searching all MS/MS spectra against a concatenated forward/reversed target/decoy version of a human Uniprot 2022 protein sequence database. The HCD-MS/MS spectra were searched with a static modification of carboxymethyl-Cys and variable modifications of oxidation (M), acetylation (protein N-terminus), and trypsin enzyme specificity required. Search parameters for the Andromeda search engine were set to an initial precursor ion tolerance of 20 parts per million and MS/MS tolerance at 0.02 Dalton. Label-free peptide quantification based on extracted ion chromatograms and spectral counts and validation was performed in the MaxQuant software suite to achieve a final peptide false discovery rate < 0.01. The minimum required peptide length was set to 7 amino acids, and peptide precursors

were filtered on individual peptide mass errors after nonlinear post-acquisition recalibration. Label-free quantification normalization (Classic) was used. Match between runs was used with a Matched Time Window of 0.7 min and an alignment time window of 20 min.

Protein Differential Expression Analysis

Subjects were excluded if they had high levels of contaminants or failed to pass quality control (QC) (see principal component analysis (PCA) (Figure S1)). To ensure data quality, we used the PTXQC R package[20] and identified the PCA outliers as samples that failed several QC parameters, such as peptide intensity distribution, identification rate over retention time, peak width over retention time, match between runs (MBR) alignment, MBR ID transfer, and oversampling (MS/MS counts per 3D-peak). Following outlier removal, the remaining 80 EV (40CTRL/40MDD) and 16 bulk (8CTRL/8MDD), 12 with overlapping EV and bulk tissue proteomics, were carried forward for downstream analysis (Table S2). Prior to analysis, potential contaminants identified by MaxQuant were removed. Proteins detected in at least 75% of subjects per group were included for downstream analysis. After median normalization, counts were imputed to account for missing values. Differential protein expression was assessed using a generalized linear model implemented in the limma package.[21] The model was adjusted for sex, pH, age at death, and postmortem interval (PMI) for bulk data, and for sex, batch, pH, age at death, and PMI for EV data. Analyses were adjusted for pH, age at death, and PMI due to their high contribution to the top 5 principal components in each dataset. The Benjamini-Hochberg method was used to correct for multiple testing, and the significance threshold was set at an adjusted *P*-value (padj) ≤ .05.

Heatmap

Normalized LFQ intensity values of EV and bulk proteomics from subjects with both information (Table S2) were first log-transformed and used for heatmap generation. Hiplot (ORG)[22] (Plugin version v0.2.4—Inter-heatmap) was used with the following parameters: cluster method = ward.D2, row and column distance measure = Euclidean, scale = row.

Correlation Matrix

Log-transformed normalized LFQ intensity values of EV and bulk proteomics from subjects with both information (Table S2) were used to generate the correlation matrix. Hiplot (ORG)[22] (Plugin version v0.1.0—Corrplot) was used with the following parameters: cluster method = ward.D2; correlation method = Pearson; order method = hclust. The significance threshold was set at $P \leq .05$.

Functional Protein Association Network

Proteins that were determined to be significantly different between MDD vs CTRL (padj ≤ 0.05) were inserted into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING)[23] version 12.0. The protein-protein interaction network was then generated.

Gene Ontology Enrichment Analysis

Significant proteins (padj ≤ 0.05) were inserted into Hiplot (ORG)[22] (Plugin version v0.2.0—GO-KEGG), and gene ontology (GO) enrichment analysis was performed via clusterProfiler[24] for biological process, molecular function, and cellular component with a *P*-value cutoff of.01 and q-value (Benjamini-Hochberg) cutoff of 0.05. To determine the percentage of proteins falling under vesicle-related terms, we input the list of all proteins identified in EVs into FunRich (3.1.3)[25-27] and performed a cellular component GO enrichment analysis.

RESULTS

ACC Proteomic Profiles Differ Between Bulk Tissue and EVs

The workflow of this study is shown in Figure 1. To determine the relationship between the proteome of EVs and that of bulk tissue from where EVs were extracted, we first analyzed the corresponding proteomes using mass spectrometry and LFQ. When comparing the relative abundance of proteins detected in at least 75% of subjects per group, we observed different proteomic profiles in EVs compared to bulk tissue (Figure 2A, Table S3). Additionally, while EV protein levels showed high correlation across subjects (correlation coefficients $0.91 \leq r \leq 1$; $P \leq .05$), there was low correlation between EV and bulk tissue protein levels of the same subject (correlation coefficients $0.1 \leq r \leq 0.18$; $P \leq .05$) (Figure 2B).

Brain EV Protein Changes Observed in Depression are Associated With Synaptic Function

To determine whether brain EV protein cargo is altered in MDD, we performed differential expression analysis comparing MDD to CTRL. We found 70 proteins to be significantly differentially expressed (padj ≤ 0.05) in brain EVs from MDD subjects, where 48 proteins were increased and 22 were decreased (Figure 3, Table 1, and Table S4). On the other hand, we did not find any significant difference in bulk tissue proteins (Table S5).

We investigated the potential functions of the 70 differentially expressed proteins using in silico methods. We first aimed to identify whether these proteins were functionally and/or physically associated with one another using STRING. We found that 57 of 70 proteins formed an interconnected network (protein-protein interaction enrichment *P*-value < 1.0e-16) (Figure 4A). We then performed GO enrichment analysis for biological process, molecular function, and cellular component (Figure 4B-D). For biological process, terms such as "synapse organization" and "neurotransmitter transport" were enriched. For molecular function, terms that are important for synaptic function, such as "GTP binding"[28] and "glycosaminoglycan binding,"[29] along with "glutamate binding," were also enriched. For cellular component, terms such as "glutamatergic synapse," "distal axon," and "SNARE complex" were enriched. For an extensive list of enriched GO terms, see Table S6. We also investigated GO terms associated with upregulated and downregulated proteins separately and found that "cell adhesion" and "immunoglobulin domain" were associated with downregulated proteins specifically. Since we found several indications that the proteins of interest might be involved in synaptic functions, we aimed to further corroborate this using a GO enrichment tool specific to the synapse, called SynGO (1.1).[30] We identified the enrichment of several synaptic terms under both biological process and cellular component (Figure S2, Table S7). Finally, to ensure that the enrichment of synaptic terms is not a result of overrepresentation of synaptic vesicles rather than EVs, we checked the proportion of well-defined synaptic vesicle proteins,[31] compared to that of the vesicles in our entire EV protein dataset. We found that only a small percentage of genes related to synaptic vesicles (1.9%) compared to the majority of proteins that related to exosomes (60.4%) (Figure 4E).

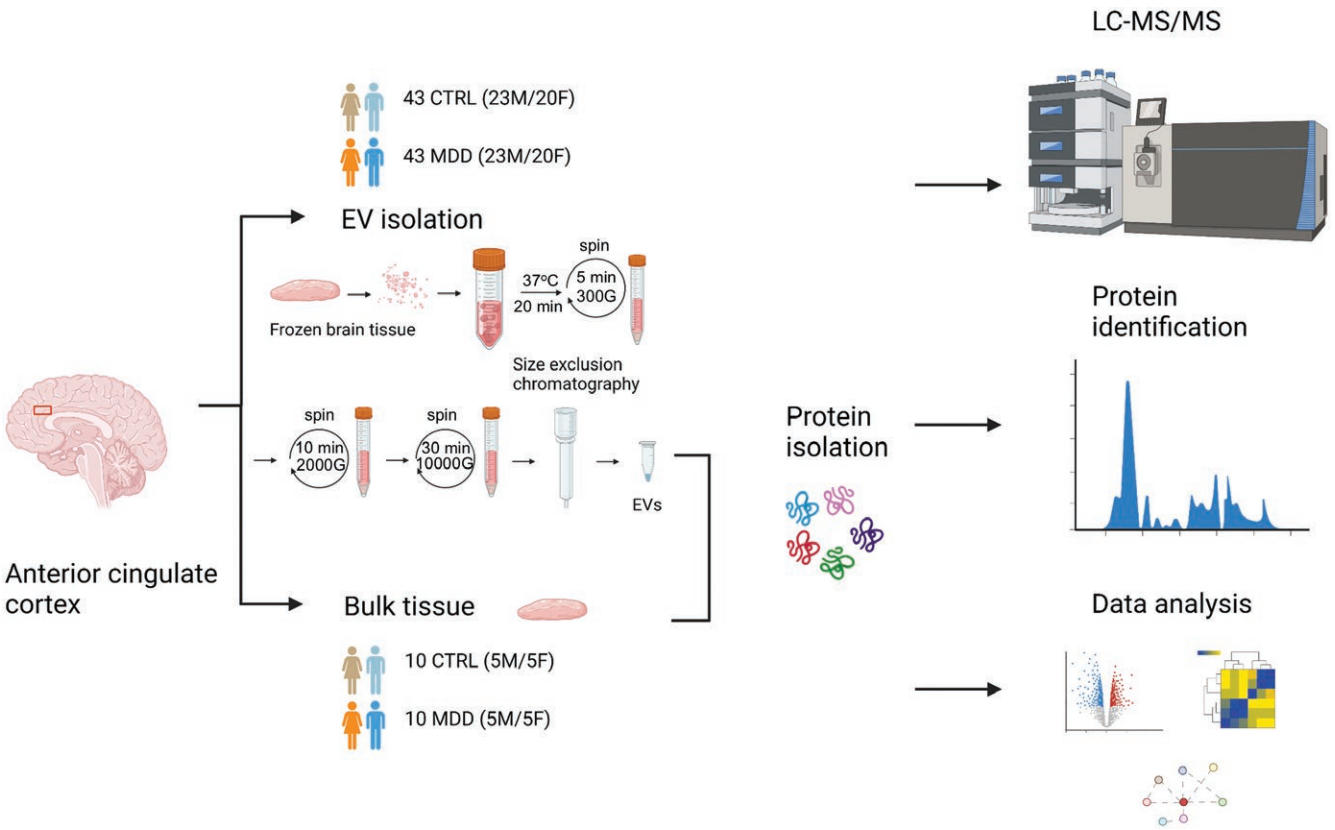

Figure 1. Schematic diagram representing study workflow. CTRL, control; EV, extracellular vesicle; F, female; LC-MS/MS, liquid chromatography tandem mass spectrometry; M, male; MDD, major depressive disorder. Created with BioRender.com.

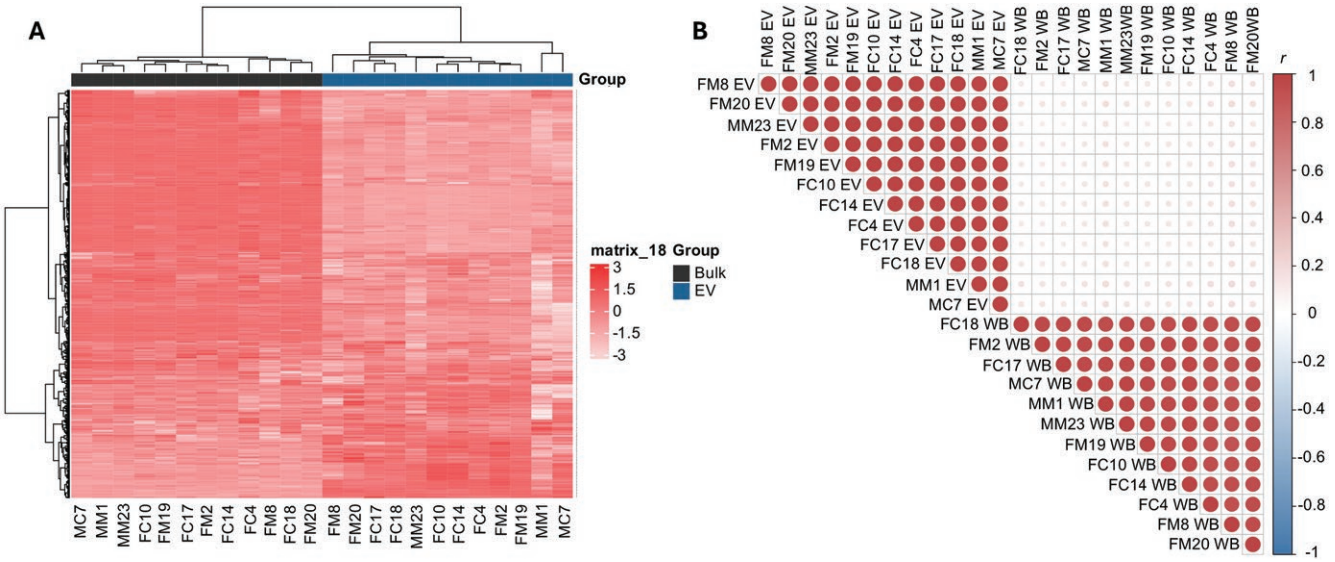

Figure 2. Comparison of proteomic profiles of EVs and corresponding bulk tissue. (A) Heatmap showing the relative abundance of proteins identified in EV and bulk tissue samples. (B) Correlation plot between EV and corresponding bulk tissue sample protein levels. EV, extracellular vesicle; FC, female control; FM, female MDD; MC, male control; MM, male MDD; WB, whole brain.

DISCUSSION

In this study, we used ultra-high-performance liquid chromatography and tandem mass spectrometry and LFQ to identify and quantify brain EV proteins of MDD and CTRL subjects. Comparative analysis revealed distinct proteomic profiles between proteins packaged into EVs and their corresponding levels in the tissue of origin (Table S3), supporting the notion that the molecular signal from EVs is distinct from that of bulk tissue. Additionally, the higher correlation between subjects compared to within subject, when considering EV and bulk tissue proteomics, further supports these findings (Figure 2B). However, we acknowledge that these results might be confounded by the

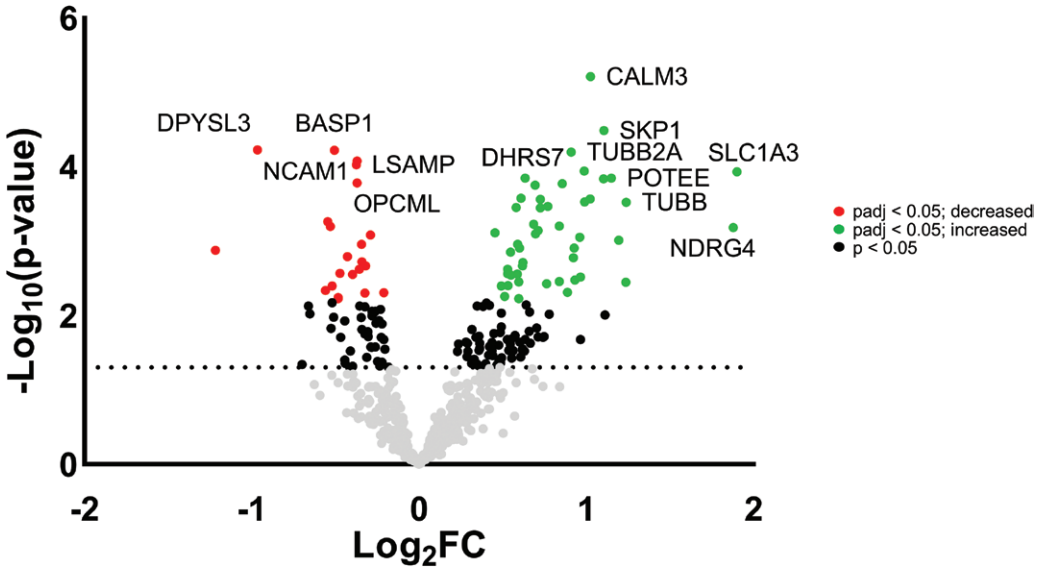

Figure 3. Differential expression analysis of EV proteins in MDD vs control. Volcano plot displays proteins that are nominally significant (do not pass correction for multiple testing) as black dots, as well as proteins that pass correction for multiple testing. Green dots: increased in MDD; Red dots: decreased in MDD. Names of top differentially regulated proteins are displayed. Padj: adjusted *P*-value after correction for multiple testing. Graph was generated using GraphPad Prism (8.0.1). EV, extracellular vesicle; FC, fold change; MDD, major depressive disorder.

small sample size used for bulk tissue. Having more statistical power might have identified more similarities between EV and bulk proteomics. Nonetheless, our data show that EVs still provide distinct information given certain protein enrichments only found in EVs, consistent with the notion that proteins are specifically sorted into EVs.[32]

Overall, we identified 70 differentially expressed EV proteins between MDD and CTRL EVs, including many previously associated with MDD (Figure 3). For instance, the glutamate transporter SLC1A3 is important for glutamatergic signaling and was downregulated in the hippocampus of MDD subjects.[33] CALM3, which is also important for glutamatergic synaptic activity, has been shown to be dysregulated in bipolar I disorder and in MDD.[34-36] BASP1 regulates the actin cytoskeleton and in turn modulates axon outgrowth and synaptic functions.[37] A negative association has been found between BASP1 antisense 1 RNA, which can regulate BASP1, and right tail hippocampal volume in MDD.[38] The neural cell adhesion molecule, LSAMP, has a prominent role in neurite formation and synaptogenesis, and polymorphisms in its gene have been linked to MDD.[39,40] NDRG4 plays a role in vesicle docking and nodes of Ranvier organization, and its gene exhibits reduced DNA methylation in adolescents with MDD.[41,42] Moreover, the DHRS7 gene was mapped to a significant locus in an early-onset subtype-specific MDD genome-wide association study,[43] and DPYSL3 was found to be differentially expressed in postmortem brains of MDD subjects.[44] While evidence from the literature points to a role for many of our differential proteins identified in EVs, none of these studies profiled proteins from EVs.

The GO terms enriched in our in silico follow-up analysis are consistent with a possible involvement of EVs, through their protein cargo, in the pathophysiology of MDD, as these terms are relevant to synaptic processes directly and indirectly (Figure 4B-D, Figure S2), and there is substantial literature suggesting that synaptic dysfunction is at the basis of most molecular perturbations in MDD (for a review, see Fries et al.[3]). In addition, there are data suggesting that EVs play a crucial role in synaptic activity.[45,46] Among the most promising GO terms, guanosine triphosphate (GTP) binding and GTPase activity are known to be involved in the morphogenesis and plasticity of dendritic spines through modulating actin filament organization.[47,48] Glycosaminoglycan binding also has a known role in synaptic plasticity.[29] In addition, cell adhesion molecules of the immunoglobulin superfamily are related to synaptic function,[49] and their downregulation has been implicated in stress-related mood disorders and antidepressant action as well.[50]

In our previous study, the most significant evidence was found for 2 microRNAs (miRNAs), miR-92a-3p and miR-129-5p, which were decreased in brain EVs of MDD subjects. Consistent with the observations of the current study, these 2 miRNAs regulate genes involved in neurotransmission and synaptic plasticity.[19] A study by Martins-de-Souza[44] performed shotgun data-independent label-free LCMS on postmortem dorsolateral prefrontal cortex (DLPFC) brain tissue, comparing MDD subjects with controls. They reported distinct proteome fingerprints between MDD and control subjects, including proteins associated with synaptic function.[44] Interestingly, among those proteins, DPYSL3, ATP6V1F, TUBB2A, ARF1, and FXYD6 were also part of our list of 70 differentially regulated proteins in EVs. A more recent study identified 295 differentially expressed proteins in the prefrontal cortex when comparing individuals who died by suicide to controls. Among those 295 proteins, 7 were in common with our findings (SYN2, PSAP, SH3GLB2, RHOB, SYNPR, TUBB4A, and DPYSL3), and the biological functions enriched were also associated with neurotransmission and synaptic signaling.[51] Stelzhammer et al.[52] performed a similar study but in pituitary tissue and found that MDD subjects also exhibit distinct profiles with cell-to-cell signaling as the most over-represented molecular function. It must be noted, however, that in this study, only 17.4% of the subjects had MDD, which might partially explain the small overlap with our study. Furthermore, Qi et al.[53] performed proteomic profiling of the ACC and DLPFC of elderly MDD subjects and found that GO terms related to synaptic function were found to be suppressed in the ACC of MDD subjects. Other studies have conducted proteomic analyses on the postmortem brain tissue of individuals who died by suicide, albeit not in MDD specifically, and found a dysregulation in proteins related to synaptic processes.[54,55] As

Table 1. Differentially regulated proteins in brain EVs of MDD subjects.

Upregulated proteins				
Uniprot ID	**Gene name**	**Log2FC**	***P*-Value**	**Adjusted *P*-Value**
P0DP25	CALM3	1.03	.000006	.0035
P43003	SLC1A3	1.90	.000116	.0067
Q6S8J3	POTEE	1.15	.000142	.0067
E5RJR5	SKP1	1.11	.000033	.0067
H0YHC3	NAP1L1	1.10	.000145	.0067
Q9Y394	DHRS7	0.99	.000114	.0067
Q13885	TUBB2A	0.91	.000063	.0067
A0A087X0J3	CAP2	0.86	.000168	.0067
F5H5D3	TUBA1C	0.69	.000178	.0067
Q68DU8	KCTD16	0.64	.000142	.0067
Q5JP53	TUBB	1.24	.000299	.0085
Q16864	ATP6V1F	1.02	.000272	.0085
C9J9E2	CAMKV	0.99	.000296	.0085
Q9HCH3	CPNE5	0.73	.000273	.0085
Q96CX2	KCTD12	0.61	.000265	.0085
Q53GQ0	HSD17B12	0.77	.000341	.0088
J3QRS3	MYL12A	0.73	.000355	.0088
Q92777	SYN2	0.58	.000354	.0088
Q9ULP0	NDRG4	1.88	.000659	.0134
Q9BPX5	ARPC5L	0.84	.000629	.0134
P37235	HPCAL1	0.69	.000592	.0134
Q9H0Q3	FXYD6	0.71	.000717	.0141
M0R0Y2	NAPA	0.70	.000799	.0147
O95741	CPNE6	0.45	.000774	.0147
F8WE43	SYNPR	0.96	.000889	.0153
P62745	RHOB	1.19	.000967	.0162
P43007	SLC1A4	0.59	.001109	.0176
P04350	TUBB4A	0.93	.001234	.0186
P59998	ARPC4	0.60	.001237	.0186
P84077	ARF1	0.55	.001401	.0200
E9PLF1	GSTM2	0.92	.001676	.0227
Q7L099	RUFY3	0.62	.001949	.0253
Q9Y2Q0	ATP8A1	0.62	.002160	.0268
P10909	CLU	0.53	.002397	.0285
Q99747	NAPG	0.59	.002761	.0308
C9JFZ1	SYNJ1	0.53	.002732	.0308
O15020	SPTBN2	0.55	.002866	.0308
P30050	RPL12	0.97	.003022	.0319
O95670	ATP6V1G2	0.93	.003318	.0344
Q5BJH1	PSAP	1.24	.003582	.0352
Q9H1V8	SLC6A17	0.84	.003484	.0352
Q9NZN3	EHD3	0.60	.003531	.0352
F5GZV7	VAMP1	0.76	.003738	.0361
P47755	CAPZA2	0.53	.003966	.0370
P68371	TUBB4B	0.49	.004026	.0370
P02792	FTL	0.89	.004874	.0431
B7ZC39	SH3GLB2	0.51	.005521	.0470
Q6VY07	PACS1	0.60	.005929	.0485
Downregulated proteins				
Uniprot ID	**Gene name**	**Log2FC**	***P*-Value**	**Adjusted *P*-Value**
Q14982	OPCML	−0.37	.000164	.0067
P13591	NCAM1	−0.37	.000083	.0067
H3BLU2	LSAMP	−0.37	.000094	.0067

Table 1. Continued

Downregulated proteins				
Uniprot ID	**Gene name**	**Log2FC**	**P-Value**	**Adjusted P-Value**
P80723	BASP1	−0.51	.000060	.0067
Q14195	DPYSL3	−0.96	.000059	.0067
O14594	NCAN	−0.55	.000547	.0130
Q96GW7	BCAN	−0.53	.000636	.0134
Q12860	CNTN1	−0.29	.000831	.0148
B7Z1Z5	NTM	−0.34	.001100	.0176
P24821	TNC	−1.22	.001328	.0194
P22748	CA4	−0.43	.001605	.0223
A0A7P0TAW3	VCP	−0.34	.001893	.0251
J3KPS3	ALDOA	−0.32	.002148	.0268
F6U236	PACSIN1	−0.36	.002395	.0285
A0A7I2V3U0	ACO2	−0.40	.002810	.0308
P10915	HAPLN1	−0.47	.002703	.0308
Q15286	RAB35	−0.52	.003982	.0370
A0A7I2V2G2	HSPA9	−0.56	.004608	.0417
Q08722	CD47	−0.21	.004918	.0431
P29966	MARCKS	−0.32	.004997	.0432
P12532	CKMT1A	−0.48	.005690	.0477
P15104	GLUL	−0.48	.005952	.0485

Abbreviations: EVs, extracellular vesicles; MDD, major depressive disorder.

mentioned earlier, our modest sample size may have confounded our ability to detect differences in bulk tissue. Although some of the abovementioned studies have comparable sample sizes, it is important to note that different brain regions and/or different subject characteristics (eg. elderly, died by suicide without MDD) were investigated. In addition, these studies showed that in depression, some proteins are dysregulated in brain tissue, while our study shows that similar proteins are dysregulated in EVs. This is not surprising given that we found both consistent and divergent protein trends between bulk and EV proteomics, suggesting that EVs may still reflect levels of some but not all proteins in bulk tissue. Moreover, our results indicate that synaptic dysfunction in MDD, which has been reported in previous literature, is also reflected in brain EVs.

Our study is not without limitations. First, Given the enrichment of multiple synapse-related terms, it is possible that we co-isolated synaptic vesicles with our EVs. Along with the evidence presented earlier, synaptic vesicles range in size from 30 to 50 nm, whereas the EVs we isolated range from 30 to 200 nm.[19] It has also been shown that it is not unusual to find synaptic proteins in EVs.[56] Nonetheless, we cannot completely exclude the idea that synaptic vesicles might have been co-isolated in our samples, but if present, they are most likely a small minority. Given the nature of the tissue used, self-assembled membranous particles formed from cell bursting might create pseudo-EVs, which may have also been co-isolated with the EVs. In addition, it is possible that we isolated both intracellular and extracellular EVs. However, we assume that pseudo-EVs and intracellular EVs are minimal since our isolation protocol is based on mild tissue dissociation using the gentle enzyme collagenase III and shaking. Moreover, contaminant proteins that represent pseudo-EVs were absent in our EV isolation.[19] We have also previously found that tissue quality, reflected by PMI, does not affect the presence of these contaminant proteins in EV isolations (Figure S3). Second, although removed bioinformatically, contaminant proteins identified in our samples might still have had an impact on the identification of lowly abundant proteins.[57] We were also unable to distinguish EV proteins from ambient proteins present in the suspension. Therefore, caution must be taken when selecting targets for downstream analyses, as future experiments must first confirm the presence of selected proteins on or within EVs. We did not investigate sex differences to maintain statistical power, but future efforts should also aim to determine whether EV proteins in depression differ by sex. Finally, we looked at EVs originating from bulk ACC tissue, so future studies should also try to refine which cell type(s) are responsible for the dysregulated signal carried in EVs. In addition, similar profiling should be conducted in other brain regions to determine whether our results are region-specific or not.

In summary, to our knowledge, this is the first study to profile proteins in EVs from postmortem brain tissue in the context of depression. Our results improve our understanding of proteomic changes in EVs of the depressed brain, which may have important implications for identifying novel therapeutic targets and biomarkers. Finally, our findings encourage and warrant future studies that investigate brain EV cargo in other mental disorders, as this will help us better understand the unique molecular signatures of depression and suicide as well as those in common with other disorders.

Supplementary material

Supplementary material are available at *International Journal of Neuropsychopharmacology* (IJNPPY) online.

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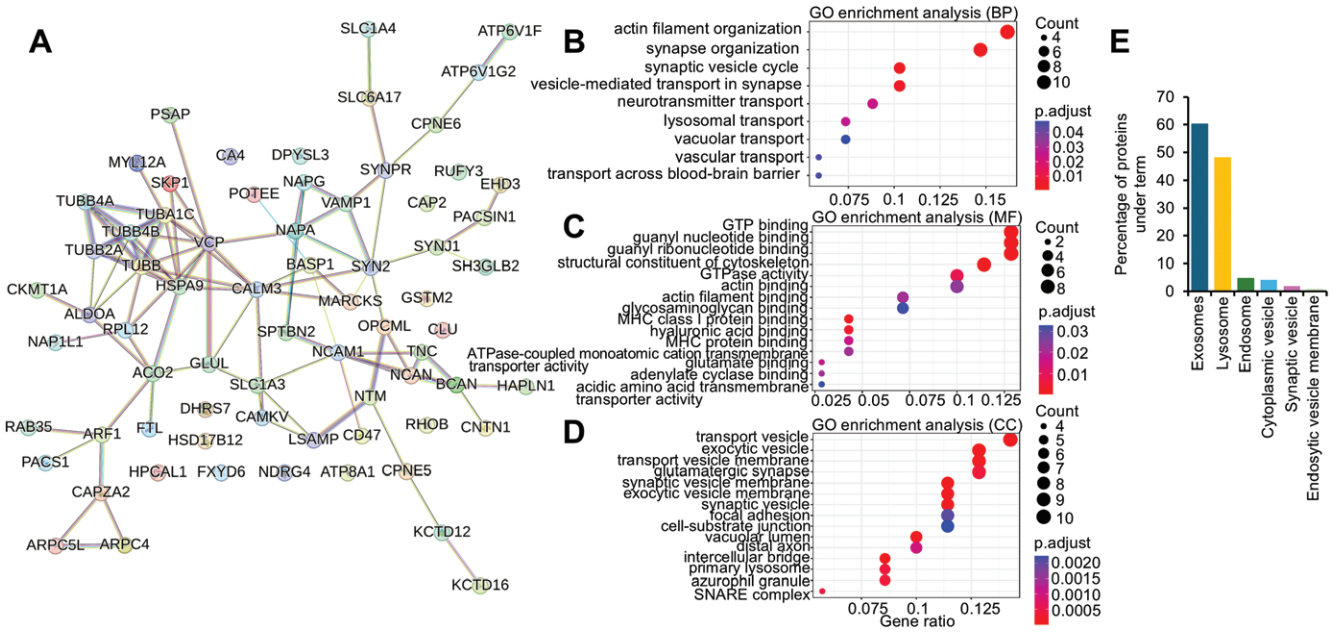

Figure 4. In silico functional follow-up on differentially packaged proteins in MDD. (A) STRING protein-protein interaction network of the 70 differentially regulated proteins. Gene ontology enrichment analysis of the 70 differentially regulated proteins for (B) biological process (BP), (C) molecular function (MF), and (D) cellular component (CC). (E) Histogram showing the percentage of proteins contributing to cellular component GO terms related to different vesicular entities. GO, gene ontology; MDD, major depressive disorder.

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Author contributions

Pascal Ibrahim (Conceptualization [lead], Formal analysis [lead], Investigation [lead], Methodology [lead], Project administration [lead], Visualization [lead], Writing—original draft [lead], Writing—review & editing [lead]), Haruka Mitsuhashi (Formal analysis [equal], Investigation [equal], Visualization [equal], Writing—review & editing [supporting]), Lorne Taylor (Data curation [equal], Methodology [supporting], Writing—review & editing [supporting]), Jenna Cleyle (Data curation [equal], Writing—review & editing [supporting]), Naguib Mechawar (Resources [equal], Writing—review & editing [supporting]), Corina Nagy (Conceptualization [equal], Project administration [equal], Supervision [lead], Writing—review & editing [supporting]), and Gustavo Turecki (Conceptualization [equal], Project administration [equal], Resources [equal], Supervision [lead], Writing—review & editing [supporting]).

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Conflicts of interest

None declared.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding authors.

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