Archival Report

Biological Psychiatry

Sustained Molecular Pathology Across Episodes and Remission in Major Depressive Disorder

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ABSTRACT

BACKGROUND: Major depressive disorder (MDD) is a debilitating mental illness and a major cause of lost productivity worldwide. MDD patients often suffer from life-long recurring episodes of increasing severity, reduced therapeutic response, and shorter remission periods, suggesting the presence of a persistent and potentially progressive pathology.

METHODS: Subgenual anterior cingulate cortex postmortem samples from four MDD cohorts (single episode, $n = 20$; single episode in remission, $n = 15$; recurrent episode, $n = 20$; and recurrent episode in remission, $n = 15$), and one control cohort ($n = 20$) were analyzed by mass spectrometry–based proteomics ($n = 3630$ proteins) combined with statistical analyses. The data was investigated for trait and state progressive neuropathologies in MDD using both unbiased approaches and tests of a priori hypotheses.

RESULTS: The data provided weak evidence for proteomic differences as a function of state (depressed/remitted) or number of previous episodes. Instead it suggested the presence of persistent MDD effects, regardless of episodes or remitted state, namely on proteomic measures related to presynaptic neurotransmission, synaptic function, cytoskeletal rearrangements, energy metabolism, phospholipid biosynthesis/metabolism, and calcium ion homeostasis. Selected proteins (dihydropyrimidinase-related protein 1, synaptosomal-associated protein 29, glutamate decarboxylase 1, metabotropic glutamate receptor 1, and excitatory amino acid transporter 3) were validated by Western blot analysis. The findings were independent of technical, demographic (sex or age), or other clinical parameters (death by suicide and drug treatment).

CONCLUSIONS: Collectively, the results provide evidence for persistent MDD effects across current episodes or remission, in the absence of detectable progressive neuropathology.

Keywords: Bioinformatics, Major depressive disorder, Mass spectrometry, Progressive neuropathologies, Remission, Subgenual cingulate cortex

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Major depressive disorder (MDD) is a severe mental disorder with heterogeneous clinical symptoms, including prominent emotion dysregulation, low mood, poor cognition, and comorbid anxiety (1,2). Globally, the World Health Organization cites MDD as the leading cause of years lost due to disability (3), reflecting the fact that for most patients MDD is a life-long illness characterized by recurring episodes, often of increasing symptom severity, longer duration with shorter and/or partial remission periods, and increasing resistance to antidepressants (4,5). An increase in the frequency and duration of depressive episodes is suggested to enhance vulnerability to additional relapses and accelerate disease progression, leading to worsening functional deficits (6). This disease trajectory suggests the presence of progressive neuropathologies where outcomes (7) and treatment efficacy (8,9) are inversely correlated with disease severity, as measured by the number and/or length of depressive episodes (10).

The subgenual anterior cingulate cortex (sgACC) has previously been implicated in both acute sadness and antidepressant treatment effects, which is suggestive of a critical role in modulating negative mood states (11,12). Patients with treatment-resistant depression were observed to have a metabolically overactive sgACC whose elevated activity could be modulated by deep brain stimulation that resulted in sustained remission of depression (13). Previous transcriptome analyses of postmortem hippocampus and temporal and prefrontal cortices showed dysregulation of messenger RNA (mRNA) transcripts involved in presynaptic neurotransmission, synaptic function, and cytoskeletal rearrangement of neuronal processes (14–18). Particularly, the alterations in presynaptic neurotransmission were associated with dysregulated gamma-aminobutyric acid (GABA) and glutamatergic receptor signaling (15–18). Reduced expression of somatostatin and other dendritic-targeting GABA neuron markers were observed in postmortem dorsolateral prefrontal cortex (DLPFC), sgACC, and amygdala from MDD subjects, in correlation with reduced brain-derived neurotrophic factor/tyrosine receptor kinase B signaling (19–22). These latter molecular studies are consistent

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with proton magnetic resonance spectroscopy (23–25) and transcranial magnetic stimulation (26) analyses demonstrating decreased inhibitory GABA levels and functions in MDD subjects (27,28). These findings suggest an altered excitation/inhibition balance in MDD that is mediated by both GABA and glutamate dysregulations (15,29).

Although transcriptomic studies have advanced our knowledge of the neurobiology of MDD, they provide only one facet of molecular changes associated with complex neuropsychiatric disorders. Mass spectrometry (MS)–based proteomics, which assays protein concentrations at lower dynamic range than the protein abundances in a cell compared to measures of mRNA levels by transcriptomic studies (30,31), is increasingly used to survey variations in protein levels during health and disease states (32–34). It is therefore well suited for unbiased discovery of alterations in protein levels associated with neuropsychiatric disorders. Previous proteomic studies performed in postmortem brains of MDD subjects have indicated enrichment of proteins involved in energy metabolism (35,36), synaptic function (35), myelination (37), and presynaptic glutamatergic neurotransmission (36). These studies were limited by the lack of more specific distinction between MDD disease states and traits, by the relatively few proteins being investigated (i.e., 1422, 56, and 1310, respectively), or by small sample size (n = 36, 44, and 46, respectively) (35–37). Western blot analysis provided evidence of reduced glutamate decarboxylase 1 (GAD-67) protein levels, a GABA synthesizing enzyme (38).

We applied MS-based proteomics supported by bioinformatics and statistics to perform the first large-scale protein investigation (n = 3630) of biological changes in MDD, more specifically in the sgACC of four MDD cohorts at various stages of disease and in one cohort of control subjects (Figure 1). We focused on the sgACC based on robust clinical and imaging evidence of deregulated function in the area (11,13) and on previous findings from our group suggesting a more severe molecular phenotype in this brain region compared with other investigated areas (21). We tested for the presence of persistent pathological findings across all MDD patients regardless of disease state (hypothesis 1), for distinct neuropathologies corresponding to current episodes or remission states (hypotheses 2 and 3), and for evidence of progressive neuropathology in association with recurrent episodes (hypothesis 4).

METHODS AND MATERIALS

Detailed methods are available for review in the Supplemental Methods in Supplement 1.

Human Postmortem Brain Samples

Postmortem sgACC (Brodmann area 25) samples were obtained through the University of Pittsburgh Brain Tissue Donation Program with consent from the next of kin. Sample collection was performed during routine autopsies performed at the Allegheny County Medical Examiner's Office (Pittsburgh, PA) with procedures approved by the University of Pittsburgh Institutional Review Board and the Committee for Oversight of Research and Clinical Training Involving the Dead. Consensus DSM-IV diagnoses were made by an independent committee of experienced clinicians using information from structured interviews with family members, clinical records, toxicology results, and standardized psychological autopsies (39). The same approach was used to confirm the absence of a psychiatric diagnosis in comparison subjects. The DSM-IV diagnosis is at time of death, whereas psychosis history is lifetime. Ninety samples including control subjects and four MDD cohorts were analyzed (Figure 1, Table 1, and Table S1 in Supplement 2). The MDD cohorts were closely matched with control subjects to ensure that they did not differ in mean age, postmortem interval (PMI), brain pH, or RNA integrity number (RIN) (Table 1). Samples comprising all six cortical layers were harvested from coronal sections, as previously described (40).

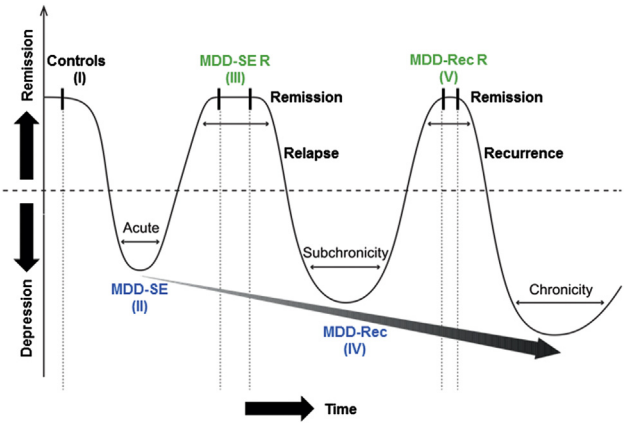

Figure 1. Overview of major depressive disorder (MDD) cohorts and control subjects used in the study. Hypothesized progressive model of MDD showing recurring episodes of increasing severity, reduced therapeutic response, and shorter remission periods. MDD episodes and treatment phases are indicated by valleys and crests, respectively. A gray arrow depicts the predicted trajectory of MDD pathology across various diseases stages. Groups are indicated by I–V. MDD-Rec, MDD recurrent; MDD-Rec R, MDD recurrent remission. MDD-SE, MDD single episode; MDD-SE R, MDD single episode remission; [Reproduced with permission from Sibille and French (52)].

Sample Preparation and Liquid Chromatography Tandem MS Analysis

Total sample homogenates (approximately 20 μg) were subjected to a modified filter-aided sample preparation protocol as previously described (41), with additional precipitation using an equal volume of 2 M potassium chloride for depletion of residual detergents. Lys-C and tryptic peptides were combined and processed on Pierce C18 Tips reversed phase resin (Thermo Fisher Scientific, Waltham, MA) for desalting and concentration. Peptides were applied to an ultra-performance liquid chromatography system (Easy-nLC 1000; Thermo Fisher Scientific) and separated on a 50-cm column (75 μm inner diameter) packed with PepMap rapid separation liquid chromatography C18 resin (Thermo Fisher Scientific) at 60°C, using a flow rate of 250 nL/min on a 5%–30% acetonitrile in 0.1% formic acid gradient over 224 minutes. Column washes were performed on a 30%–90% acetonitrile in 0.1% formic acid gradient for 2 minutes, then 12 minutes in 90%

Table 1. Characteristic Features of MDD Cohorts and Control Subjects Used in the Study

Variables	Group 1: Controls (n = 20)	Group 2: MDD-Single Episode (n = 20)	Group 3: MDD-Single Episode in Remission (n = 15)	Group 4: MDD-Recurrent Episode (n = 20)	Group 5: MDD-Recurrent Episode in Remission (n = 15)
Age, Years, Mean	47.9	42.3	47.6	40.9	48.1
Sex, % Male	90	80	53	65	67
PMI, Mean	15.35	15.99	12.17	16.8	16.4
pH, Mean	6.76	6.62	6.63	6.62	6.61
RNA Ratio	1.55	1.62	1.69	1.57	1.42
RIN, Mean	8.09	8.08	8.45	8.03	8.03
Suicide, %	0	50	0	40	0
Antidepressant Use ATOD, %	5	60	20	75	60

Postmortem brain samples from all cortical layers of the subgenual cingulate cortex included: control subjects and four MDD cohorts as described above. Most subjects in the groups are white: controls (75%), single episode (80%), single episode remission (93%), recurrent (85%), and recurrent remission (93%). There were no significant differences between the groups with regard to mean age, PMI, pH, RIN, or RNA ratio. However, the groups varied in sex ratios, suicide rates, and antidepressant use ATOD.

ATOD, at time of death; MDD, major depressive disorder; PMI, postmortem interval; RIN, RNA integrity number.

acetonitrile in 0.1% formic acid. Peptides were introduced into an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) using a nanoelectrospray ion source (Easy-Spray; Thermo Fisher Scientific) (Figure S1 in Supplement 1). Mass spectra were acquired in a 400–1200 m/z range with a resolution of 240,000 at 400 m/z in the Orbitrap (automatic gain control target, 100,000), followed by 10 data-dependent MS/MS scans (automatic gain control target, 10,000). The top 10 most intensive ions were selected and fragmented by collision-induced dissociation with normalized collision energies of 30 in the ion trap. Dynamic exclusion duration was set at 50 seconds and the maximum exclusion list size at 500.

MS Data Analysis

Raw MS data were processed by MaxQuant software (version 1.5.3.8) and searched against the human UniProt database (released November 2015; 20,193 entries), for label-free quantitation of peptides and proteins. The following settings were used: fragment ion mass tolerance of 20 parts per million, maximum two missed cleavages (trypsin and Lys-C), fixed modification as carbamidomethylation of cysteine, and variable modification as oxidation of methionine and acetylation of protein *N*-terminal. False discovery rates were set at 1% for both peptide and protein levels in target/decoy to minimize false positives. The match between runs feature was used. Reverse sequences, potential contaminants, and those only identified by site (Table S2-1 in Supplement 3) were removed from the MaxQuant data before statistical analysis. This processing yielded 4301 proteins (Table S2-2 in Supplement 3) for further analysis.

Statistical Analysis

Data points at 0 were added a value of 1, before log2 transformation of processed MS intensity data from MaxQuant. Data imputation was performed to address the observed 0 values (real zeros or missing data), a common procedure in proteomics data (42). Zero values were imputed based on three criteria: proportion of samples with nonzero intensity in each group, the molecular weight of the protein, and the mean intensity over all samples. Consequently, we classified all proteins into five categories: 1) complete data (proportion of nonzero equal to 1 in both control and all MDD groups); 2) mild missing but imputable data (proportion of nonzero $\geq$0.7 in both groups); 3) moderate missing and nonimputable data (proportion of nonzero $<$0.7 in exactly one group); 4) severe missing and noninterpretable data (proportion of nonzero $<$0.7 in both groups, both its molecular weight and mean intensity are above the 33rd percentile in all proteins); and 5) severe missing but interpretable data (proportion of nonzero $<$0.7 in both groups, and its molecular weight or mean intensity is below the 33rd percentile in all proteins). Only the zeros in category II were imputed using the k-nearest neighbor method (k = 10 in our case). We accepted the zeros in all the other categories. Downstream analyses were based on proteins from categories 1 (2421 proteins) and 2 (1209 proteins), in order to ensure high confidence in our data. Proteins from categories 3 (144 proteins), 4 (0 proteins), and 5 (527 proteins) that were of low confidence were not analyzed further (Table S2-3 in Supplement 3). Differentially expressed label-free quantified proteins were identified using the random intercept model (RIM) with parameter selection employing the smallest Bayesian information criterion (43) to account for potential covariates (adjusting for up to two cofactors among age, pH, PMI, and RIN). To correct the potential bias of the variable selection procedure, we performed a permutation analysis that randomly shuffled the disease labels within each pair to generate a null distribution for p value assessment (B = 500). For the exploratory purpose of this study, we used $p \leq .05$ and $\geq$20% fold change (RIM coefficient $\geq \pm 0.26$) as thresholds for differentially expressed proteins. Post hoc analysis to correct for the potential confound effects of psychosis, alcohol dependence, antidepressant drug use, and death by suicide on differential protein expression was performed using analysis of variance. To test the potential of progressive effects, we hypothesized a linear relationship between MDD groups and the expression level of some proteins, across all five groups, in current episodes or in remission only. Compared with the use of categorical or nominal variable where each category is relatively independent, the ordinal approach used in this study assumes that the categories can be ordered. The five groups in

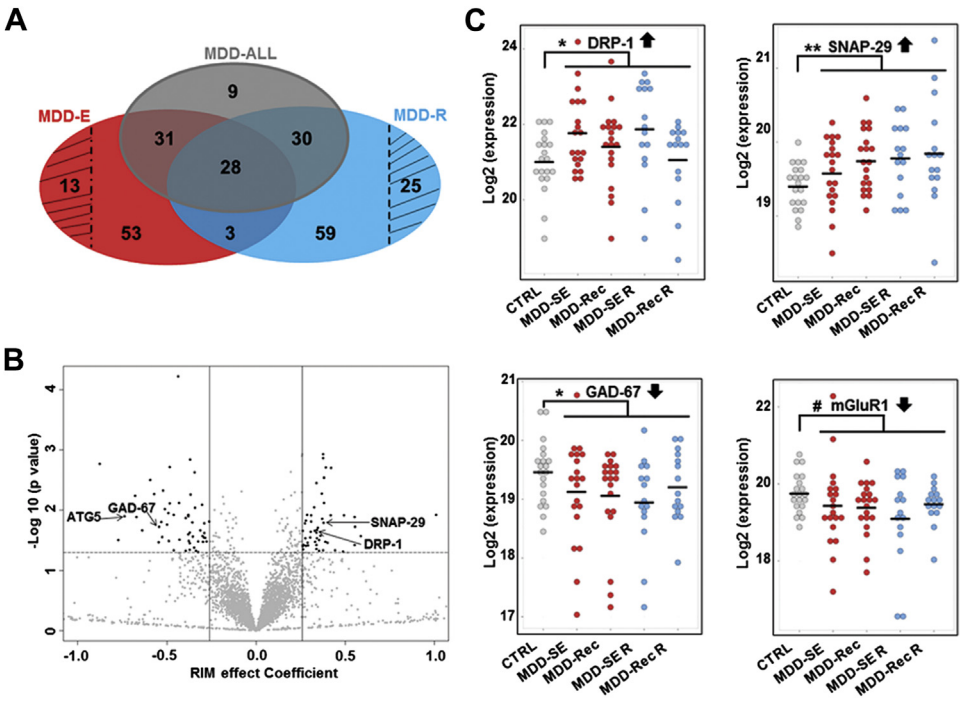

Figure 2. Summary of identified proteins across major depressive disorder (MDD) episodes and remission phases. **(A)** Venn diagram of the differentially expressed proteins associated with all MDD patients (MDD-ALL, gray shading) and those in current episodes (MDD-E, red shading) or remission phases (MDD-R, blue shading) at $p \leq .05$ and random intercept model (RIM) coefficient effect (log2 fold change) $\geq \pm 0.26$ thresholds. Differentially expressed proteins associated only with patients in current episodes ($n = 13$) or remission ($n = 25$) are indicated with black line shading. **(B)** Volcano plot indicating distribution of identified proteins based on their RIM effect coefficient (log2 fold change) and –log 10 (p value). Upregulated and downregulated proteins are highlighted in black (see right and left panels, respectively). Examples of upregulated (DRP-1 and SNAP-29) and downregulated (ATG5 and GAD-67) proteins are indicated in the plot. **(C)** Scatter plots of selected differentially expressed proteins across in subgroups of all MDD patients. CTRL, control subjects; DRP-1, dihydropyrimidinase-related protein 1; EAAT3, excitatory amino acid transporter 3; GAD-67, glutamate decarboxylase 1; MDD-Rec, MDD recurrent; MDD-Rec R, MDD recurrent remission; MDD-SE, MDD single episode; MDD-SE R, MDD single episode remission; mGluR1, metabotropic glutamate receptor 1; SNAP-29, synaptosomal-associated protein 29. #Trend, *$p \leq .05$, and **$p \leq .01$, respectively.

our data reflect progressive stages of MDD, which we assumed are ordered categories and therefore hypothesized that a linear (stage) effect on protein expression might exist.

Western Blot Analysis

Details on sample processing, protein detection, and antibodies are described in the Supplemental Methods in Supplement 1.

Functional and Network Analysis

Gene ontology analysis was performed using the Protein ANalysis THrough Evolutionary Relationships (PANTHER) database (version 11.1; http://pantherdb.org) (44). The following settings were used: analysis type, PANTHER overrepresentation test (release 20170413); annotation version and release date–gene ontology database released 2017-05-25; reference list, MDD background list of all identified proteins in ≥70% samples (n = 3630); and annotation data set—gene ontology biological process complete and no correction for multiple testing, because of the limitations in sample availability of postmortem tissues. We also probed for enrichmnet in PANTHER pathways. Differentially expressed proteins were subjected to Ingenuity Pathway Analysis (www.ingenuity.com) for canonical pathways and disease categories. The following parameters were used for core analysis: Ingenuity Knowledge Base (genes only) as a reference set, direct and indirect relationships, interaction networks, all data sources, confidence of experimentally observed data only, the species set to humans, and including data from only brain tissues. The scoring method was based on Fisher's exact test.

RESULTS

Do MDD Patients Exhibit a Persistent Disease Effect, Regardless of Current Episode or Remission State, Compared With Controls?

Characteristics of the MDD cohorts corresponding to current episodes, remission states, and control subjects are summarized in Figure 1 and Table 1. sgACC gray matter samples from postmortem brain tissues were used in liquid chromatography-MS/MS–based proteomic analyses (Figure S1 in Supplement 1), followed by label-free quantification of proteins using MaxQuant software. We detected 4301 proteins (Table S2-2 in Supplement 3), of which 3630 were identified in ≥70% of all samples (Table S2-3 in Supplement 3) and subsequently used for downstream analyses. A comparison of control subjects to all MDD patients using a RIM to correct for potential confounders yielded 98 differentially expressed proteins, at $p \leq .05$ with fold change ≥20% (Log2 ratio ≥ ±0.26) (Figure 2A, gray shading, and Table S2-4 in Supplement 3). Examples of upregulated proteins (Figure 2B) within this group include synaptosomal-associated protein 29 (SNAP-29), which is involved in autophagy/vesicle exocytosis; dihydropyrimidinase-related protein 1 (DRP-1), an axonal guidance signaling protein; and rho GTPase-activating protein 32, a protein involved in N-methyl-D-aspartate receptor activity–dependent actin reorganization in dendritic spines. Examples of downregulated proteins (Figure 2B) include GAD-67, which catalyzes the production of GABA; calcium/calmodulin-dependent protein kinase type 1G; and autophagy-related protein 5 (Table S2-4 in Supplement 3).

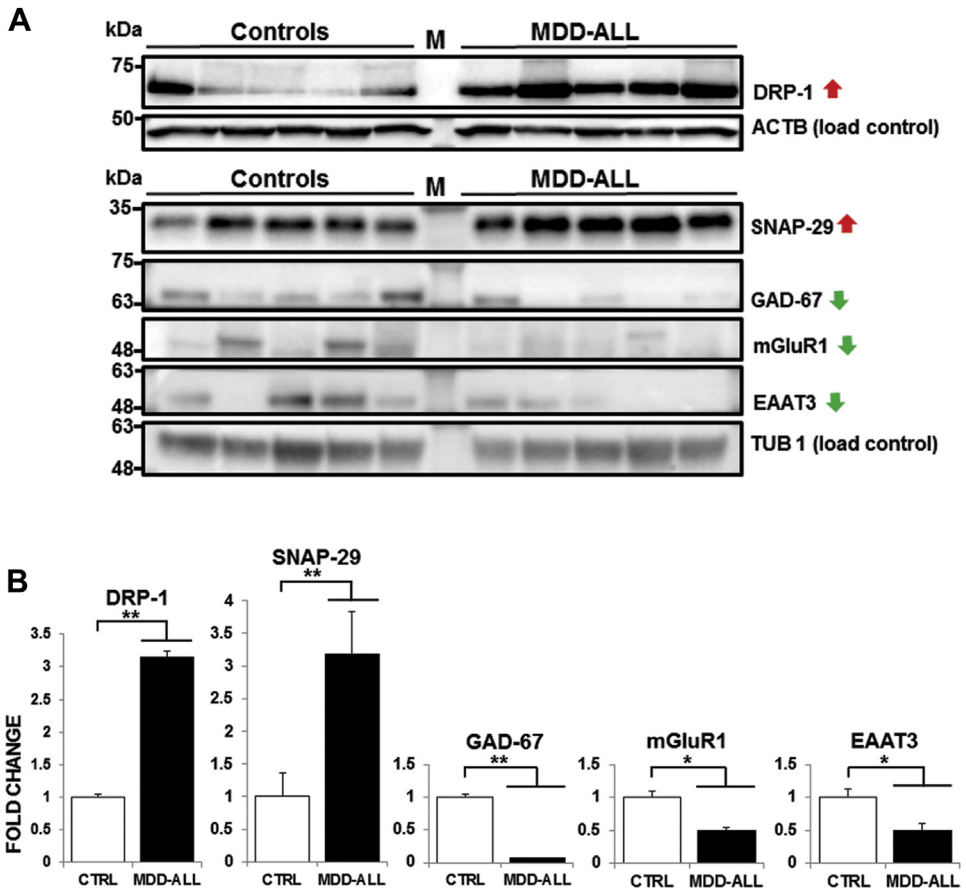

Figure 3. Western blot analysis of selected differentially expressed proteins in major depressive disorder (MDD) cohorts. **(A)** Immunoblotting with DRP-1, SNAP-29, GAD-67, mGluR1, and EAAT3 antibodies showed increased protein expression of DRP-1 (62 kDa) and SNAP-29 (29 kDa), but reduced protein expression of GAD-67, mGluR1, and EAAT3 (67, 62, and 57 kDa, respectively) for MDD patients in current episodes compared to control subjects. Anti-beta actin (ACTB; 42 kDa) and anti-beta tubulin (TUB1; 56 kDa) were used to assess equal loading of protein lysates. The two protein standards (labeled as M) used were All Blue Prestained Protein Standards (BioRad Canada) and BLUeye Prestained Protein Ladder (Thermo Fisher Scientific). **(B)** Quantitation of the bands using Image Lab software (BioRad) indicated statistical significance (*$p \leq .05$ and **$p \leq .01$). CTRL, control subjects; DRP-1, dihydropyrimidinase-related protein 1; EAAT3, excitatory amino acid transporter 3; GAD-67, glutamate decarboxylase 1; MDD-ALL, all MDD patients; mGluR1, metabotropic glutamate receptor 1; SNAP-29, synaptosomal-associated protein 29.

Selected scatter plots of two upregulated (DRP-1 and SNAP-29) and downregulated (GAD-67 and metabotropic glutamate receptor 1 [mGluR1]) proteins in the various subgroups of all MDD subjects are shown in Figure 2C.

A few differentially expressed proteins were selected for independent evaluation by Western blot analysis, on the basis of their known involvement in MDD or other psychiatric disorders (DRP-1 and GAD-67) and autophagy/vesicle exocytosis (SNAP-29). We also included proteins identified at $p < .1$ and ≥20% fold change based on their previous association with psychiatric disorders (mGluR1 and excitatory amino acid transporter 3 [EAAT3]). mGluR1, which is reduced in MDD patients and was also downregulated in our proteomic analyses at the trend level, served as an internal validation of MDD effects (38). We anticipated that proteins that were upregulated (DRP-1 and SNAP-29) or downregulated (GAD-67, mGluR1, and EAAT3) in our proteomic analyses would show increased/decreased expression in immunoblots of MDD cohorts compared with control subjects. Western blot analysis of samples from a randomly selected subset of five MDD patients and control subjects that were equally matched for mean age, PMI, brain pH, or RIN indicated immunoreactive bands corresponding to endogenously expressed DRP-1, SNAP-29, GAD-67, mGluR1, and EAAT3, respectively (Figure 3A). We determined significantly increased protein expression for DRP-1 ($p = .0081$) and SNAP-29 ($p = .0029$) and decreased protein expression for GAD-67 ($p = .0004$), mGluR1 ($p = .0296$), and EAAT3 ($p = .0471$) (Figure 3B) in the subset of the MDD patients we tested. Results of the Western blot validation are summarized in Table S3 in Supplement 1.

As a group, differentially expressed proteins associated with a persistent disease effect (at $p < .05$ and ≥20% fold change) were previously linked in the literature to neurological diseases (Ingenuity Pathway Analysis) (Table 2) and most significantly implicated in ganglioside metabolic process (66.67% overlap; fold enrichment 24.13), sequestering of metal ion (50.00% overlap; fold enrichment 18.10), protein kinase R–like endoplasmic reticulum kinase-mediated unfolded protein response (50.00% overlap; fold enrichment 18.10), and phosphatidylglycerol biosynthetic process (33.33% overlap; fold enrichment 12.07) (Table 2). These findings were independent of technical, demographic, or other clinical parameters (death by suicide and drug treatment) (Tables S2-4 to S2-11 in Supplement 3). Moreover, Ingenuity Pathway Analysis indicated phosphatidylglycerol biosynthesis II (nonplastidic; 13.6% overlap) and calcium signaling (3.5% overlap) as the two most significantly affected canonical pathways (Table 2 and Table S4 in Supplement 2). In addition, we detected proteins of previous interest based on literature knowledge, including mGluR1 and EAAT3 at the less stringent $p < .1$ threshold (Table S2-4 in Supplement 3). Together, the data support the presence of a persistent disease effect among all MDD patients that involves

Table 2. Biological Processes and Pathways Associated With All Major Depressive Disorder Patients

	Overlap	Fold Enrichment	*p* Value	Associated Proteins
GO Biological Process Term				
Ganglioside metabolic process (GO: 0001573)	66.67%	24.13	3.22E-3	HEXB and ITGB8
Sequestering of metal ion (GO: 0051238)	50.00%	18.10	5.63E-3	FTH1 and S100A8
PERK-mediated unfolded protein response (GO: 0036499)	50.00%	18.10	5.63E-3	ASNS and HEBP1
Phosphatidylglycerol biosynthetic process (GO: 0006655)	33.33%	12.07	1.22E-2	TAM41 and PTPMT1
Positive regulation of cytokine-mediated signaling pathway (GO: 0001961)	28.57%	10.34	1.63E-2	1-AGPAT 1 and CNTFR
CDP-diacylglycerol biosynthetic process (GO: 0016024)	28.57%	10.34	1.63E-2	TAM41 and 1-AGPAT 1
IPA Canonical Pathways[a]				
Phosphatidylglycerol biosynthesis II (nonplastidic)	3/22 (13.6%)	–	3.47E-4	TAM41, PTPMT1, and 1-AGPAT 1
Calcium signaling	5/144 (3.5%)	–	2.19E-3	ara CALC, CAMK1G, TPM3, TPM1, and SLC8A1
Retinoate biosynthesis II	1/1 (100%)	–	6.35E-3	RBP1

Differentially expressed proteins were determined by statistical analysis using the RIM, based on $p \leq .05$ and RIM effect coefficient $\geq \pm 0.26$ thresholds. GO biological process analysis was performed using the PANTHER database (http://pantherdb.org). IPA was used to identify the top 3 canonical pathways and significantly associated disease categories linked to the differentially expressed proteins.

CDP, cytidine diphosphate; GO, gene ontology; IPA, Ingenuity Pathway Analysis; PERK, protein kinase R–like endoplasmic reticulum kinase-mediated; RIM, random intercept model.

[a]The differentially expressed proteins were most significantly associated with neurological diseases (psychological disorders) in the disease category of Ingenuity Pathway Analysis. The overlap values indicate the ratio of identified proteins to those within the group.

dysregulation of phospholipid biosynthesis/metabolism, calcium ion homeostasis, axon guidance, and GABA or glutamate receptor signaling–related proteins.

Are Current Episodes of Depression Associated With a Unique Profile of Differentially Expressed Proteins?

We next probed for proteins that were differentially expressed exclusively in MDD patients in current episodes. For this, protein expression had to be significantly different from controls and from the group of MDD patients in remission (Figure 2A). The analysis yielded 13 proteins that are restricted to MDD patients in current episodes (Figure 2A, red with black line shading, and Table S2-7 in Supplement 3). Examples of the downregulated proteins in this group include 26S proteasome non-ATPase regulatory subunit 5 and serine/threonine-protein phosphatase 1 catalytic subunit alpha. The low number of differentially expressed proteins in this category precluded meaningful functional analysis. Together, the data provide weak evidence for the presence of a distinct molecular disease state for patients in current episodes compared with those in remission and healthy control subjects.

Are MDD Patients in Remission Associated With a Unique Profile of Differentially Expressed Proteins?

Similarly, we probed for proteins that were differentially expressed exclusively in MDD patients in remission. For this, protein expression had to be significantly different from the control subjects and from the group of MDD patients who had a current episode of MDD at the time of death (Figure 2A). The analysis yielded 25 proteins that are restricted to MDD patients in remission (Figure 2A, blue with black line shading, and Table S2-8 in Supplement 3). Examples of the upregulated proteins in this group include voltage-dependent L-type calcium channel subunit beta-1 and BTB/POZ domain-containing protein KCTD8. Among the downregulated proteins were synaptophysin and D-2-hydroxyglutarate dehydrogenase, mitochondrial. Differentially expressed proteins specific to MDD patients in remission were too few to allow for meaningful functional analysis. Overall, the data provide weak evidence in support of a distinct molecular state for the MDD patients in remission, compared with those who experienced a current episode of depression at time of death and healthy control subjects.

Additional proteins that fell into an intermediate category were identified. These proteins show significant expression changes between MDD patients in current episodes ($n = 53$) or in remission ($n = 59$) compared with control subjects without being either differentially expressed between MDD cohorts (i.e., patients in current episodes vs. those in remission) or identified in the first analysis combining all MDD subjects (Figure 2A, gray shading). The functional groups with these proteins demonstrated a considerable overlap with those corresponding to differentially expressed proteins identified in the combined cohort (i.e., either in current episodes or in remission; Table S5 in Supplement 2 and Tables S2-5 and S2-6 in Supplement 3). Therefore, although these proteins had not been identified in the combined cohort, they may be considered as belonging to the general MDD category rather than representing distinct pathologies between remission and current episodes.

Do MDD Patients Exhibit Progressive Neuropathology Across MDD Episodes and Remission Phases Compared With Controls?

Statistical analysis was performed by recoding the group variable as an ordinal variable (groups I–V) and fitting a RIM by adjusting for up to two cofactors among age, PMI, pH, and RIN to detect a possible linear effect across groups. Only three proteins fit a progressive neuropathology profile across MDD

episodes and remission phases at the less stringent $p \leq .1$ and $\geq \pm 20\%$ fold change (Table S2-9 in Supplement 3). Given this low signal, we tested for the possibility of a progressive neuropathology restricted to patients in current episodes, i.e., from control subjects to early (single episode) and late (recurrent) MDD stages. A similar analysis was performed for MDD patients in remission. Thirty-nine and 41 differentially expressed proteins were associated with progressive MDD effect for patients in current episodes and remission, respectively, based on $p \leq .05$ and fold change $\geq \pm 20\%$ (Tables S2-10 and S2-11 in Supplement 3). The differentially expressed proteins associated with the progressive MDD effect for patients in current episodes were mostly implicated in mitochondrial calcium ion homeostasis, whereas regulation of cytokine production/phosphatidylinositol 3-kinase signaling were the top functional groups linked to a similar disease effect for patients in remission (Table S5 in Supplement 2). Together, these findings provide no supporting evidence for a progressive neuropathology across episodes and remission, and only weak evidence for progressive MDD effects restricted to patients in either current episodes or remission.

DISCUSSION

Previous large-scale human postmortem studies on depression focused on the depressive state and used analysis of the transcriptome (14–18) as a proxy measure for biological function. We investigated for the first time biological changes associated with various disease states (current episode, remission, and recurrence) in the sgACC in MDD and opted for a large-scale proteomic analysis using MS-based approaches in a large postmortem cohort.

The results provide novel evidence in support of persistent disease effects in all MDD patients (regardless of depressive episodes or remission). In contrast, the data provided weak evidence in support of distinct pathological states in patients who experienced current episodes of MDD or who were in remission from the illness at time of death. Interestingly, we found even weaker evidence for a progressive disease effect, whether the subjects were analyzed together or in subgroups based on current episode or remission. These latter findings contrast with clinical evidence showing increased disease severity with recurrence. They suggest that the increasing disease severity and treatment resistance observed in patients with recurrent depression may be mediated by other factors (e.g., sex differences, regional connectivity, and neural network changes) rather than by greater molecular pathology, at least within the limits of detection of our assays. These results provide insight into disease mechanisms and progression that have implications for treatment strategies.

Analysis of differentially expressed proteins provided some insight into biological pathways associated with presynaptic neurotransmission, energy metabolism, synaptic function, and cytoskeletal reorganization (Table S2-4 in Supplement 3), in agreement with previous findings using combined cohorts (14–18,35–37). Moreover, we also determined novel enrichment in phospholipid biosynthesis and calcium signaling functional groups/pathways in these patients (Table 1 and Table S4 in Supplement 2). These findings were not attributable to the effect of sex, antipsychotic medications, or other potential confounders and therefore likely reflect the underlying disease process (Table S2-4 in Supplement 3, Tables S6 and S7 in Supplement 2, and Figures S2 and S3 in Supplement 1). Phospholipids are the main constituent of biological membranes and in neurons are critical for subcellular compartmentalization of integral membrane proteins involved in neuronal communication (45). A major role of Ca^{2+} in neurons is to regulate activity-dependent signaling by controlling neuronal excitability. Loss of neuronal Ca^{2+} homeostasis during aging has been associated with alterations in neuronal excitability and consequently changes in neuronal networks and metabolism (46).

Downregulated proteins associated with the persistent MDD pathology included GABA/glutamate receptor signaling proteins (GAD-67, mGluR1, and EAAT3) (Tables S2-4, S2-5, and S2-6 in Supplement 3), consistent with previous reports suggesting impaired gamma-aminobutyric acidergic and glutamatergic neurotransmission in MDD (15–18). Moreover, dysregulation of cytoskeletal organization by Rho GTPase is associated with altered dendritic spine morphogenesis, which has been suggested to contribute to deficits in synaptic function in MDD (47). GAD-67 was shown to be significantly reduced in the frontal cortex of depressed subjects at the protein (38) and mRNA levels (19–22), whereas reduced somatostatin mRNA expression in the dorsolateral prefrontal cortex, sgACC, and amygdala of MDD patients has previously been demonstrated (19–22). We also observed a downregulation of somatostatin, although at the trend level ($p \leq .1$), as one of the few proteins potentially associated with a progressive MDD effect for patients in remission (Table S2-11 in Supplement 3). These GABA/glutamate/synaptic results are noteworthy because they provide internal control validity since we used exploratory statistical criteria for the identification and functional annotation of differentially expressed proteins. We detected neuronal proteins, including DRP-1 and SNAP-29 (upregulated) and mGluR1 and EAAT3 (downregulated), which supports the sensitivity of our MS analyses for probing differential protein expression in human postmortem brain tissues. A subset of the neuronal proteins was further validated using Western blot analyses (Figure 3). Interestingly, upregulated DRP-1, as well as downregulated mGluR1 and *SLC1A1* mRNA transcripts, have previously been associated with schizophrenia (48–50). Future targeted MS experiments with selected reaction monitoring or parallel reaction monitoring based on a priori knowledge from this study and known MDD hypotheses should allow for more accurate and sensitive detection of lowly expressed proteins that are putatively altered at different stages of depression. Selected reaction monitoring has already been successfully used to probe for changes in protein levels of selected candidate markers for neuropsychiatric disorders (37).

Although our study provided novel and interesting insights, there were several limitations. First, MS-based analysis has a relatively lower dynamic range in comparison to the complexity of the cellular proteome (30). Second, proteomic analysis of human postmortem tissues is also challenging due to the variation in postmortem interval before autopsy, during which protein degradation may occur (51). Third, although our assay exceeded previous proteomic studies (35–37) with more than 4300 proteins identified, it was limited by sample availability

and likely underrepresentation of membrane proteins, which may have precluded the identification of receptors associated with the monoamine hypothesis of MDD. Finally, we did not measure posttranslational modifications.

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