Sustained Molecular Pathology Across Episodes and Remission in Major Depression

Supplement 1

The PDF file includes:

SUPPLEMENTARY METHODS AND MATERIALS

SUPPLEMENTARY FIGURES S1-S4

SUPPLEMENTARY TABLE LEGENDS S1-S2 and S4-S7 (tables available separately in Excel files; see Supplement 2 and Supplement 3)

SUPPLEMENTARY TABLE S3

SUPPLEMENTARY METHODS AND MATERIALS

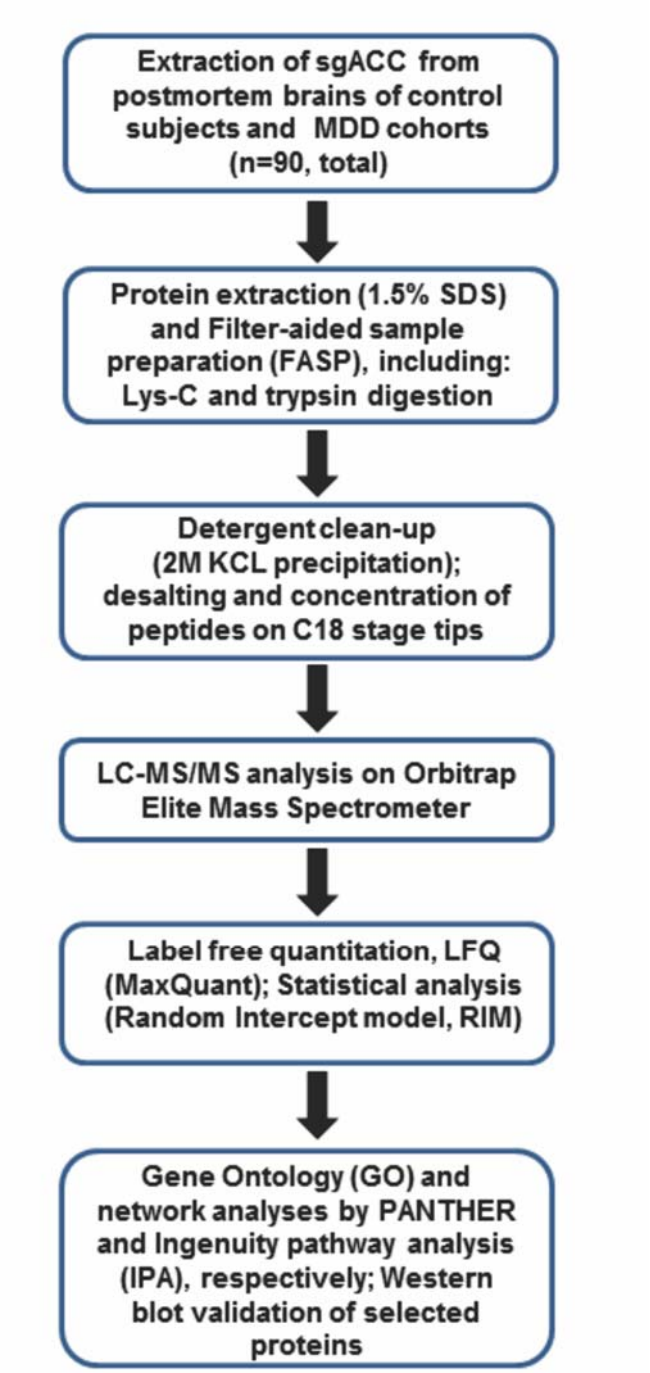
Optimization of sample preparation conditions and MS runs duration

During methods development, we performed a pilot experiment to determine the optimum conditions for subsequent MS analysis, including: detergent concentration for protein extraction, reproducibility of technical replicates and duration of MS runs. We determined that buffer with 1.5% SDS was suited to protein extraction and yet compatible with downstream MS analysis, technical replicates were found to have excellent reproducibility between both peptide/protein identifications and extracted ion current runs. Finally, 3 h MS runs were optimum for subsequent MS experiments. As such, we opted for single shot MS analysis as a compromise to increase the number of biological replicates, which potentially provide more biological insight. Samples were processed in 5 batches and there was no batch-effect on protein expression distribution (Supplementary Figure S4).

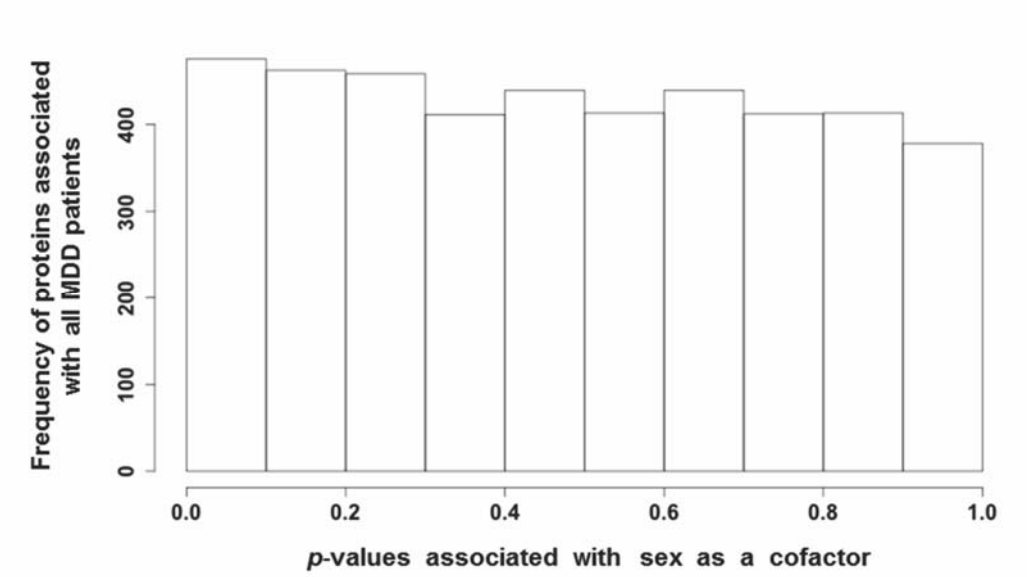
Western blot analysis

Five control and MDD patient samples were randomly selected for comparison in Western blot assay. Tissue samples were homogenized in 50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1.5 % SDS, 1 mM DTT; supplemented with: 1× protease and phosphatase inhibitor cocktail (ThermoScientific) and protein concentrations estimated by silver stain analysis using bovine serum albumin protein standards (0.1-1 µg). A total of 20 µg protein were denatured in Laemmli sample buffer at 95°C for 5 min and separated by SDS-polyacrylamide gel electrophoresis on 4-20% Criterion Stain Free™ Tris-HCl Protein gels (Biorad). Analyses were performed as using the following primary antibodies: rabbit polyclonal anti-human DRP-1 (ab137837, 1:1000); rabbit monoclonal anti-human SNAP-29 [EPR9198(2)] (ab181151, 1:1000) and rabbit polyclonal anti-human TUB1 (ab155201, 1:5000) (Abcam Inc, Toronto, Canada); rabbit polyclonal anti-human mGluR1 (PAE821HU01, 1:200) (Cloud clone/Cedarlane,

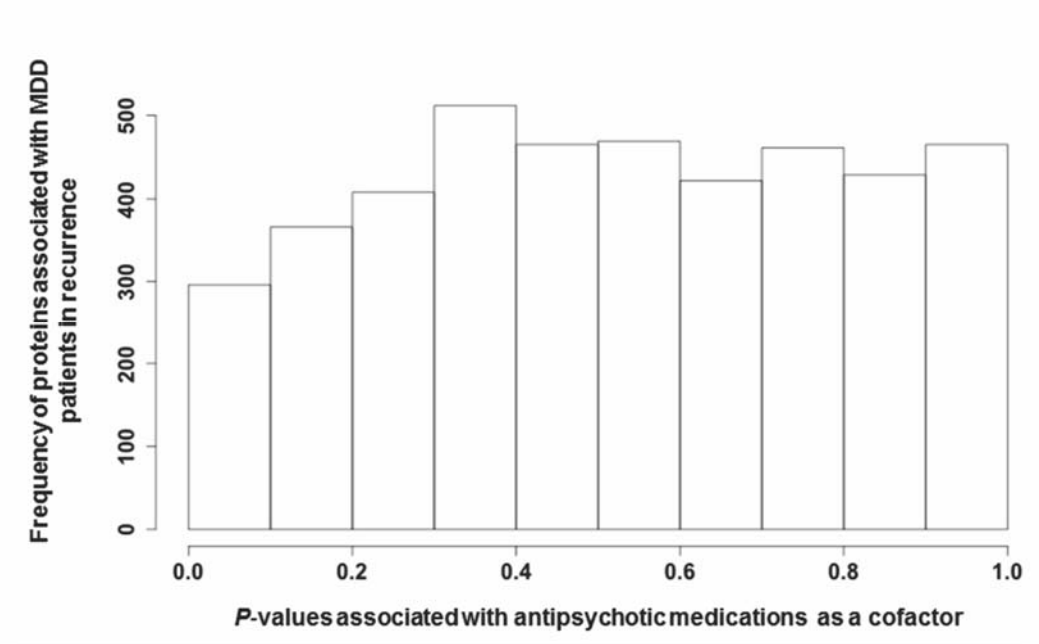
Burlington, Canada); mouse monoclonal anti-human GAD-67 (clone 1G10.2, 1:200) (EMD Millipore, Billerica, USA); rabbit polyclonal anti-human EAAT3 (SAB2102170, 1:500) and mouse monoclonal anti-β-actin [AC15] (A1978, 1:5000) (Sigma-Aldrich Canada Co., Oakville, Canada). Image acquisition and densitometric analysis of immunoblots was performed using ImageLab software version 5.2.1 (Biorad). The mean of individual MDD patient samples was used to plot fold change as a ratio to the controls. Statistical analysis of Western blots was performed using the two tailed T-test and variation in individual samples was assessed by determining the standard deviation.



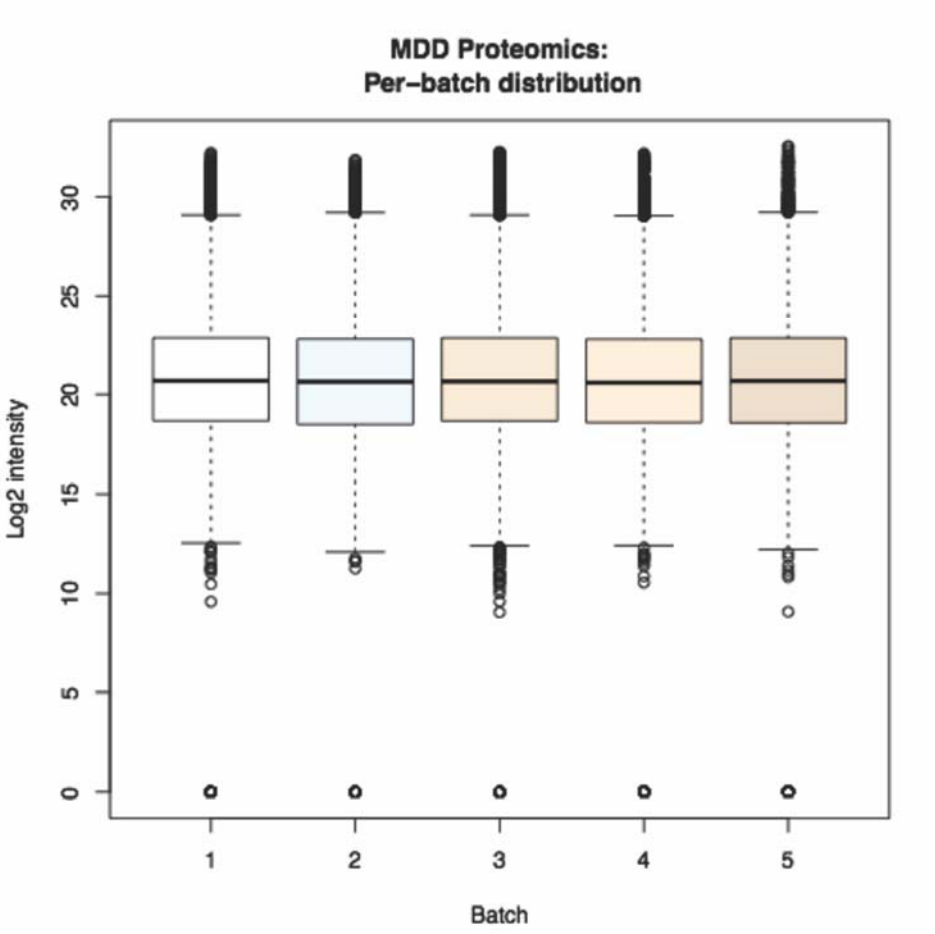
Supplementary Figure S1: MS-based proteomics workflow for analysis of sgACC gray matter. Human postmortem subgenual cingulate cortex samples from control subjects and MDD cohorts were processed using our MS-based proteomics approach. The strategy for analysis of the brain samples comprised of 4 key steps: tissue extraction, sample preparation, LS-MS/MS analysis and bioinformatics.



Supplementary Figure S2: Histogram of *p*-value distribution for identified proteins associated with all MDD patients in comparison to controls, with sex as a cofactor. The *p*-values for all the identified proteins with sex as a cofactor are evenly distributed from 0 through 1, which is very close to the distribution of *p*-value under null.



Supplementary Figure S3: Histogram of *p*-value distribution for identified proteins associated with MDD patients in recurrence in comparison to controls, with antipsychotic medications as a cofactor. The *p*-values for all the identified proteins with antipsychotic medications as a cofactor are evenly distributed from 0 through 1, which is very close to the distribution of *p*-value under null.



Supplementary Figure S4: Plot of batch effect on protein expression distribution in controls and MDD cohorts. The log2 intensity and batches are shown on the y and x-axes, respectively. The plot indicates that there is no batch effect on protein expression distribution in the samples analyzed.

SUPPLEMENTARY TABLE LEGENDS

See Supplement 2 (Excel file) for Supplementary Tables S1 and S4-S7; See Supplement 3 (Excel file) for Supplementary Table S2

Supplementary Table S1: Description of MDD cohorts and control subjects used in the study.

Postmortem brain samples were collected from control subjects (group I, n=20) and four major MDD cohorts corresponding to single episode (group II, n=20), single episode remission (group III, n=15), recurrent (group IV, n=20), and recurrent-partial/full remission (group V, n=15). DSM-IV diagnoses, mode of death (MOD), cause of death (COD) and demographic features (including: sex, age, race, PMI, pH, RNA ratio, RIN) of each subject are identified. A few control subjects with a DSM diagnosis were used and their duration of illness (DOI) is entered in parentheses (). The DOI is determined from the first depressive episode until remission or death, whichever comes first. The DSM-IV diagnosis is at time of death whereas psychosis history is lifetime. For all others subjects DOI is only for the MDD diagnosis. Abbreviations: ASCVD, atherosclerotic cardiovascular disease; ADC, Alcohol Dependence, current at time of death; ADR, Alcohol Dependence, in remission at time of death; AAC, Alcohol Abuse, current at time of death; AAR, Alcohol Abuse, in remission at time of death; ODC, Other Substance Dependence, current at time of death; ODR, Other Substance Dependence, in remission at time of death; OAC, Other Substance Abuse, current at time of death and OAR, Other Substance Abuse, in remission at time of death; Meds/Tob/Dep ATOD, Prescribed medications, tobacco use and depression at time of death; B, Benzodiazepines; C, Anticonvulsants; D, Antidepressants; N, No medications; O, Other medication(s); P, Antipsychotic; T, Tobacco; U, Unknown; V, Valproic Acid; X, Medications unknown; N/A, not applicable; 0, absence of trait; 1, presence of trait and 0/1, partial trait.

Supplementary Table S2: Analysis of MS data from MDD cohorts and control subjects by MaxQuant.

S2-1: Label free quantitation of MS data from MDD cohorts and control subjects by MaxQuant analysis-unfiltered. S2-2: Label free quantitation of MS data from MDD cohorts and control subjects by MaxQuant analysis-filtered. S2-3: Testing of hypotheses in support or not of MDD episodes and remission phases. S2-4: Differentially expressed proteins associated with a persistent disease effect in all MDD patients (hypothesis 1). S2-5: Differentially expressed

proteins associated with the MDD state of patients in current episodes (hypothesis 1a). S2-6: Differentially expressed proteins associated with the residual MDD state of patients in remission (hypothesis 1b). S2-7: Differentially expressed proteins associated with distinct MDD state for patients in current episodes in comparison to those in remission (hypothesis 2). S2-8: Differentially expressed proteins associated with distinct MDD state for patients in remission in comparison to those in current episodes (hypothesis 3). S2-9: Differentially expressed proteins associated with neuro-progressive pathology across MDD episodes and remission phases (hypothesis 4). S2-10: Differentially expressed proteins associated with a progressive MDD effect for patients in current episodes (hypothesis 4a). S2-11: Differentially expressed proteins associated with a progressive MDD effect for patients in remission (hypothesis 4b). Post hoc analysis to test for the effects of psychosis, alcohol dependence, antidepressant drug use and death by suicide on differential protein expression was performed using analysis of variance (ANOVA).

Supplementary Table S4: Canonical pathways associated with a persistent disease effect in all MDD patients (hypothesis 1).

S4-1: The pathways associated with differentially expressed (DE) proteins associated with a persistent disease effect in all MDD patients (hypothesis 1) at $p \leq 0.05$ and RIM Coefficient effect $\geq \pm 0.26$ thresholds, were identified using Ingenuity Pathway Analysis (IPA). S4-2: Pathways associated with the DE proteins in all MDD patients at less stringent $p \leq 0.1$ and RIM Coefficient effect $\geq \pm 0.26$ thresholds.

Supplementary Table S5: Overview of differentially expressed proteins associated with MDD episodes and remission phases.

Differentially expressed proteins were identified based on $p \leq 0.1$ and RIM Coefficient effect $\geq \pm 0.26$ thresholds. The biological significance of differentially expressed proteins in the MDD cohorts and association with disease categories were explored by GO biological process using PANTHER and Ingenuity Pathway Analysis (IPA), respectively.

Supplementary Table S6: Post hoc analysis of identified proteins associated with all MDD patients in comparison to controls, with adjustment of one cofactor at a time. Generated *p*-values associated with individual cofactors served as a measure of their respective importance. This analysis indicated that sex had little effect on the *p*-value of identified proteins

in comparison to the other cofactors, e.g. pH and Age, which we selected and adjusted through the random intercept model (RIM).

Supplementary Table S7: Post hoc analysis of identified proteins associated with MDD patients in recurrence in comparison to controls, with adjustment of one cofactor at a time. This analysis indicated that antipsychotic medications had little effect on the *p*-value of identified proteins in comparison to the other cofactors, e.g. pH and Age, which we accounted for in the random intercept model (RIM).

Supplementary Table S3: Summary of Western blot validation of selected differentially expressed proteins.

Selected differentially expressed proteins (DRP-1, SNAP-29, GAD-67, mGluR1 and EAAT3) in all MDD patients based on MS analysis, were validated by Western blot analysis. The directionality of protein changes observed by Western blot analysis was consistent with findings from the MS data. DRP-1 and SNAP-29 were upregulated, whereas GAD-67, mGluR1 and EAAT3 were observed to be downregulated in both analyses.

Protein name	LC-MS/MS Analysis			Western Blot Validation		
	Direction	*p*-value*	RIM effect coefficient	Direction	*p*-value*	STDEV
DRP-1	+	3.02E-2	0.59	+	8.09E-3	7.70E-2
SNAP-29	+	1.04E-2	0.33	+	2.94E-3	6.53E-1
GAD-67	_	4.96E-2	-0.37	_	4.42E-4	5.92E-3
mGluR1	_	6.99E-2	-0.41	_	2.96E-2	4.55E-2
EAAT3	_	9.62E-2	-0.43	_	4.71E-2	1.05E-1

*Correlation between the *p*-values from the LC-MS/MS analysis and Western blot validation was 0.88, indicating a strong positive correlation

Abbreviations: LC-MS/MS, liquid chromatography–tandem mass spectrometry; RIM, random intercept model; STDEV, standard deviation.