Comprehensive post mortem brain samples analysis detects global reduction of multiple proteasome subunits expression in schizophrenia

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Abbreviations

SMRI, Stanley Medical Research Institute; MSSM , Mount Sinai School of Medicine; superior temporal gyrus, STG; Ubiquitin mediated proteasome system, UPS; Post-mortem interval, PMI

ABSTRACT

Background: Ubiquitination is a common post-translational modification, responsible for proteasomal degradation, the main intracellular protein degradation system. Recently, aggregations of ubiquitinated proteins have been identified in schizophrenia. While disruption of proteasome activity is a potential cause, search for such disruption in schizophrenia has yielded inconsistent results.

Methods: We performed transcriptome sequencing of 14 postmortem superior temporal gyrus (STG) samples of subjects with schizophrenia and 15 matched controls from the Stanley Medical Research Institute (SMRI), and compared differential expression (schizophrenia vs. control) and pathway enrichment analysis to that of an independent cohort from the Mount Sinai School of Medicine (MSSM). Meta-analysis of differential expression was applied to 39 proteasome subunits genes. Replicability was tested on six additional independent datasets of four additional brain regions.

Results: The two independent STG cohorts showed high replicability. Pathway enrichment analysis of the schizophrenia down-regulated genes pointed to proteasome-related pathways. 12 out of 39 proteasome subunit genes were found to be down-regulated in schizophrenia. The signal of down-regulation of multiple proteasome subunits in schizophrenia was replicated in six additional datasets.

Conclusions: We detect global down-regulation of multiple proteasome subunits in schizophrenia. The concordance between 8 independent cohorts (overall 267 schizophrenia and 266 control samples) remarkably strengthens the validity and robustness of our results. The observed down-regulation might lead to proteasome dysfunction that can be the cause of the recently detected aggregation of ubiquitinated proteins in schizophrenia.

1 Introduction

Schizophrenia, a debilitating illness affecting 1% of the population, has a complex pathophysiology that is still far from being fully understood. Recently, the ubiquitin mediated proteasome system (UPS), a protein degradation system, has been associated with schizophrenia. The UPS was shown to be dysregulated at the transcript (1–4) and protein levels (5, 6) in brain samples of subjects with schizophrenia, with a tendency for down-regulation. In addition, UPS peripheral blood transcript levels have been associated with severity of positive symptoms (7). The UPS was associated with schizophrenia at the genomic level too - a copy number variant meta-analysis revealed that two ubiquitin related gene-ontologies were highly enriched with schizophrenia associated copy number variants (8), and the proteasome pathway was enriched in the group of schizophrenia susceptibility genes (9).

Recent findings suggest a more pronounced role of the UPS in the pathogenesis of schizophrenia. Ubiquitinated protein levels were found to be elevated in the orbitofrontal cortex of patients with schizophrenia compared to controls (10). Similarly, individuals with treatment-resistant schizophrenia had higher levels of ubiquitinated proteins in erythrocytes compared to those with recent disease onset (10). Searching for a possible mechanism, no change has been detected in (10) in proteasome activity between schizophrenia and control, in neither blood nor brain. Another study (11), however, did find intra-cellular compartment-specific dysfunction of the proteasome activity in STG samples patients with schizophrenia from the Mount Sinai School of Medicine (MSSM) cohort. Finally, increase in protein insolubility and increased ubiquitination in the insoluble fraction has been found in (12), in a subset of brain samples of patients with schizophrenia, suggesting that sequestration of misfolded proteins plays a role in its pathogenesis, possibly through disruption of critical

pathways. Thus, while elevation of ubiquitinated protein levels seems to play a role in schizophrenia, it is not clear whether this is caused by dysfunction of the proteasome. Of note, decreased proteasome activity is generally associated with ubiquitinated protein accumulation (13), as has been recently detected in schizophrenia.

Several studies reported differential expression of proteasome subunits in schizophrenia (2, 4, 14, 15), but agreement between them was sporadic, with only two subunits reported as differentially expressed in more than a single study. These two were PSMA1 (also named (20S α1), a structural subunit, which was found to be down-regulated in both (2) and (16); and PSMC6 (also named 19S Rpt4), a regulatory particle subunit, which was reported as down-regulated by (2) and (4). Only two studies (5, 11) have examined protein levels of proteasome subunits in schizophrenia**,** with three regulatory subunits found to be down-regulated in both. However, both studies examined elderly subjects (mean age 70+), from the same brain bank, MSSM, and only a subgroup of proteasome subunits were measured. Thus, while there is evidence for down-regulation of both mRNA and protein levels of proteasome subunits in schizophrenia, the results are sporadic and inconsistent.

Turning back to the transcriptome, note that differential expression studies of human brain samples of patients with schizophrenia generate both false positives and false negatives and the results of one study are often not reproduced by another. A basic limitation is the cellular complexity of the brain tissue and the fact that brain samples are usually composed of a mixture of different cell types. This might cause a situation where authentic gene expression changes in a subpopulation of the cells are diluted and reported as false negatives. In addition, as schizophrenia is highly heterogeneous both

genetically and clinically (17), we expect only modest changes in gene expression between groups of patients and controls. Indeed, it has been shown that the magnitude of fold change is typically modest (less than 1.33) and thus difficult to detect (18). A plausible and a relatively simple way to deal with these limitations is to perform a systematic comparison between results of separate, independent gene expression datasets. However, only few studies of schizophrenia have used gene expression data from more than one source (for example, (18)). To the best of our knowledge no study has performed transcriptome analysis of independent datasets of post mortem STG samples of patients with schizophrenia. Here we performed RNA sequencing of the STG of 14 schizophrenia and 15 control subjects from the SMRI cohort. The STG participates in the development of auditory hallucinations (19, 20) and the volume of this cortical area is decreased in subjects with schizophrenia (21). We identified a group of differentially expressed genes that served as input to pathway enrichment analysis, to search for pathways that are dysregulated in schizophrenia. We then used an independent cohort of elderly subjects with schizophrenia from the MSSM brain collection to test the consistency and robustness of our results. A systematic meta-analysis, integrating both SMRI and MSSM, was applied to a subgroup of 39 genes, which were shown to be both highly inter-connected and with a clear tendency to down-regulation in schizophrenia. We then used 6 additional independent gene expression datasets of different brain regions to further check the replicability and robustness of our results. Note that one of the six datasets was from the same patients as the SMRI data described above, but from a different brain region.

2 Methods and Materials

2.1 Subjects

Stanley Medical Research Institute (SMRI) subjects

STG postmortem tissues from 15 subjects with schizophrenia and 15 healthy controls were obtained from the SMRI using approved protocols for tissue collection and informed consent (22). All samples were examined by a certified neuropathologist to exclude Alzheimer's disease and other cerebral pathology (23). Diagnoses were performed independently by two psychiatrists according to DSM-IV criteria, and matched by age, sex, post-mortem interval (PMI) and pH (Table 1). RNA sequencing was applied to 29 out of the 30 STG samples (one sample did not pass quality control – see below).

Mount Sinai School of Medicine (MSSM) subjects

Human brain samples of 19 schizophrenia and 14 matched healthy controls from the STG were obtained from the Brain Bank of the Department of Psychiatry of the MSSM (Table 1). All cortical dissections and sample preparation were described previously (24–26). Brain banking activities were approved by the MSSM Institutional Review Board and written consent for brain donation was obtained from the next-of-kin of all subjects. cases diagnosed as schizophrenia met the DSM-III/IV criteria, as determined by clinical investigators. None of the samples, of neither subjects with schizophrenia nor controls, showed evidence of any significant neuropathology (27). Whole-genome gene expression was measured using Affymetrix HG-U133A microarrays.

Table 1. Subjects' characteristics; Average values (standard deviation)

Characteristics	Schizophrenia	Control	P-value
SMRI subjects			
Number of subjects	14	15	
Gender (M/F)	9/5	9/6	0.82
Age (years)	43.6 (13)	48.1 (10.6)	0.32
Brain pH	6.2 (0.3)	6.3 (0.2)	0.35
PMI (minutes)	2052 (900)	1424 (596)	0.034
MSSM subjects			
Number of subjects	19	14	
Gender (M/F)	14/5	5/9	0.03
Age (years)	77.4 (10.9)	82.4 (12.7)	0.23
Brain pH	6.4 (0.2)	6.6 (0.3)	0.08
PMI (minutes)	814 (499)	460 (429)	0.04

2.2 RNA sequencing

The brain regions were dissected by the staff at SMRI, and total RNA was isolated in Sheba Medical Center, Israel, using the Trizol method. Total RNA samples were delivered on dry ice to The Nancy & Stephen Grand Israel National Center for Personalized Medicine (G-INCPM) for quality control and whole transcriptome sequencing. The concentration of total RNA and RNA Integrity Number value (RIN) were measured. Samples with concentration of $\geqslant$ 10 ng/μl and RIN $\geqslant$5 were selected for sequencing, with 29 out of 30 samples passing (one schizophrenia sample was excluded). Among these samples, the mean RIN was 6.3 (± 0.5), and the mean ratio of 260/280 was 1.6 (± 0.14). The mean total RNA yield was 15.4 μg (± 9.7).

Libraries preparation was done using the INCPM-RNA-seq protocol (see supplementary methods for a description of the protocol). For raw RNA sequencing data description see Table 1S.

2.3 Mapping, quantification of gene expression levels and pre-processing

We used standard software tools for mapping fragments to the genome and for quantification of gene expression levels. See supplementary methods for full description. Pre-processing: Lowess correction was calculated (28). Then expression threshold was set to 6 (log scale) to reduce noise. Filtering: Genes with expression values below 6 in at least 80% of the samples were filtered out of the analysis. 16,482 genes were left for the rest of the analysis after filtering (out of 23,715).

2.4 MSSM microarray pre-processing

Standard MAS-5 algorithm was used for normalization. Lowess correction was then applied, expression levels below 20 were set to 20 and log2-transformation was done. Probe-sets without assigned Affymetrix gene symbols annotation were removed. 12,033 probe-sets were left for the rest of the analysis after filtering (out of 22,283), representing 8,542 gene symbols. Probe sets of the same gene were combined. For full details see supplementary methods.

2.5 Differential gene expression analysis

We fitted a linear model to estimate the effects of the technical and sample covariates that were provided for both MSSM and SMRI datasets, as listed in Table 1. As PH did not differ significantly between the schizophrenia and control samples (see Table 1), we included the other three covariates, age, gender and post-mortem interval (PMI). We used a stepwise procedure (29) for the linear fit for each gene separately, using the MATLAB function stepwiselm, with the default parameters. Then, we refitted the

model using only the selected variables, including diagnosis. Finally, for each gene, the diagnosis coefficient was statistically tested for being nonzero, implying an estimated effect for schizophrenia, above and beyond any other effect from the covariates. This test produces a t-statistic and a corresponding P value. P values were adjusted for multiple hypothesis testing using the false discovery rate (FDR) procedure (30). Since the differential gene expression in brain samples of subjects with schizophrenia versus controls was previously shown to be modest (18) and the list of differentially expressed genes is subjected to further pathway enrichment analysis, we decided to use a non-stringent FDR threshold of 15%. We performed also a standard 2-sided t-test on the expression values in schizophrenia versus controls. The results were very similar to those obtained by linear regression (see Figure 1S in supplementary information).

2.6 Pathway enrichment analysis using GeneAnalytics

GeneAnalytics tool (geneanalytics.genecards.org (31)) was applied to compute enrichment of biological pathways. GeneAnalytics leverages PathCards (http://pathcards.genecards.org/), which clusters thousands of pathways from multiple sources into Superpathways, in order to improve inferences and pathway enrichment analysis and reduce redundancy. Superpathways were scored based upon log2-transformation of the binomial *p*-value which was equivalent to a corrected p-value with significance defined at <0.05.

2.7 Differential expression STRING database network view

Network creation: Given a list of genes, a network is built. A network consists of genes (nodes) and genes co-expression relations (edges). The co-expression relations data was downloaded from the STRING database, version 10.5 (32). Each such connection has a score between 0 and 1 that "indicates the estimated likelihood that a given interaction is biologically meaningful, specific and reproducible" (32). The

product of this process is a network whose nodes correspond to genes and edges corresponds to co-expression relations, where the edges' weights are the STRING score. Only edges with score greater than 0.1 are considered.

Differential expression network view: Given a network and gene expression data, of both patients and controls, the following steps are taken, for each gene:

1) The mean expression and standard deviation values, Mc and Sc, are calculated using the controls samples only.

2) The mean expression, Mp, is calculated using the patients' samples.

3) Mp-Mc is calculated, the difference in the expression mean between the two groups of samples.

4) The deviation from the control group is calculated, by: (Mp-Mc)/Sc

Then the network is displayed as an undirected graph such that the node's colors correspond to the deviation described above, (Mp-Mc)/Sc. The edges represent co-expression relations. Note that only genes that have co-expression relations with other genes in the network are displayed.

3 Results

3.1 UPS related pathways are enriched in the group of genes which are down-regulated in SMRI STG samples of individuals with schizophrenia

Differential expression analysis was performed, yielding 881 up-regulated and 986 down-regulated genes. In order to examine possible connection to antipsychotic medications, alcohol or substance use, we performed correlation analyses between the expression pattern of the differentially expressed genes and Fluphenazine equivalent dosage, substance use and alcohol use measures. Correlation analyses for Fluphenazine equivalent dosage and alcohol use did not reveal any significant association with differential expression. Correlation analysis for substance use

detected two down-regulated genes (out of 986) with statistically significant correlated expression (see supplementary methods and Figures 2S-4S).

Pathway enrichment analysis was applied separately to the up and down-regulated genes. Results are presented in Table 2 (Table 1S) for the down-regulated (up-regulated) genes. It can be seen that out of 49 enriched pathways, five are directly UPS related (appear in bold in Table 2). While several pathways have higher enrichment scores, we focus on the UPS related pathways, since five such pathways were enriched, and several additional pathways, closely related to UPS, were also enriched (see Table 2).

3.2 The UPS signal is highly replicated in the MSSM STG samples

We tested whether our findings are replicated in the STG of the MSSM cohort, an independent cohort of elderly subjects. We first examined whether the two datasets are comparable. Though microarrays, which were used in the MSSM, differ from RNA-seq in their capture features, there was a high positive correlation of the t-statistics (schizophrenia vs. control) between SMRI and MSSM across all 7,498 genes common to both platforms (Figure 1A).

We next repeated the differential expression and pathway enrichment analyses in the MSSM cohort. 919 genes and 794 genes were found to be up-regulated and down-regulated in schizophrenia, respectively. MSSM and SMRI differentially expressed genes have a significant overlap – corresponding to hypergeometric P-values of $9.8*10^{-7}$ and $1.1*10^{-19}$ for the up-regulated and down-regulated genes, respectively (see Figure 1B).

Table 2. Pathway enrichment analysis of down-regulated genes with corrected p-value < 0.05. GeneAnalytics tool superpathways that were found to be enriched in the list of down-regulated genes are ordered by descending order of their enrichment score. The enrichment scores are in the second column and the superpathways' names are listed in the third column. The fourth column presents the number of down-regulated genes that belong to each superpathway , with the total number of genes of the superpathway in parentheses. MSSM enrichment score is given in the 5th column, where (-) sign means that the superpathway wasn't enriched in the list of MSSM down-regulated genes. For superpathways that are known to involve the UPS, a reference indicating the UPS involvement is given in the 6th column. Ubiquitin-proteasome directly related pathways are in bold.

#	Score	SuperPath Name	Num Matched (SuperPath) genes	MSSM Enrichment Score	Evidence for UPS involvement
1	41.07	MRNA Splicing - Major Pathway	65 (307)	33.59	
2	28.4	Chks in Checkpoint Regulation	46 (224)	18.43	
3	27.72	Translational Control	41 (189)	24.61	
4	26.05	Vesicle-mediated Transport	93 (660)	18.05	
5	25.02	CDK-mediated Phosphorylation and Removal of Cdc6`	114 (880)	18.12	the UPS plays a central role (33)
6	24.58	Gene Expression	203 (1841)	29.46	
7	21.52	Protein Processing in Endoplasmic Reticulum	34 (166)	20.01	integrally involved in the UPS (34)
8	21.33	DNA Damage	49 (292)	13.79	closely involve the UPS (35)
9	21.24	Cell Cycle, Mitotic	84 (622)	13.65	tightly regulated by the UPS (36)
10	**19.58**	**Ubiquitin-Proteasome Dependent Proteolysis**	**27 (122)**	**18.4**	
11	**19.06**	**Metabolism of Proteins**	**175 (1628)**	**25.5**	
12	18.59	Regulation of Degradation of DeltaF508 CFTR in CF	18 (63)	10.93	dominated by the UPS (37)
13	16.6	Cell Cycle	28 (145)	-	
14	**16.59**	**Ubiquitin Mediated Proteolysis**	**27 (137)**	**10.94**	
15	16.23	Signaling By Hedgehog	27 (139)	12.4	
16	**15.57**	**Proteolysis_Putative Ubiquitin Pathway**	**12 (35)**	**-**	
17	15.39	Cellular Response to Heat Stress	20 (89)	13.16	heat shock proteins recognize misfolded proteins and incorporate the UPS (38)
18	15.35	Transcription-Coupled Nucleotide Excision Repair (TC-NER)	23 (112)	-	
19	15.29	Nucleotide Excision Repair	16 (61)	-	
20	14.6	Class I MHC Mediated Antigen Processing and Presentation	95 (823)	15.89	
21	14.52	Innate Immune System	210 (2132)	22.32	
22	14.01	HIV Life Cycle	98 (865)	11.41	
23	13.78	Clathrin-mediated Endocytosis	25 (137)	-	a key process that

					transports a wide range of molecules from the cell surface to the interior and is closely regulated by the UPS (39)
24	13.68	Signaling By NOTCH1	22 (113)	-	
25	13.47	Copper Homeostasis	14 (54)	-	
26	13.46	Mitotic G1-G1/S Phases	27 (156)	-	
27	13.29	Transport to The Golgi and Subsequent Modification	41 (285)	17.23	
28	13.2	Regulation of Cholesterol Biosynthesis By SREBP (SREBF)	14 (55)	-	
29	13.13	Telomere C-strand (Lagging Strand) Synthesis	20 (100)	-	
30	13	Terpenoid Backbone Biosynthesis	11 (36)	-	
31	12.61	Processing of Capped Intronless Pre-mRNA	10 (31)	-	
32	12.56	Mitotic Metaphase and Anaphase	29 (180)	11.56	tightly regulated by the UPS (40)
33	12.45	Transport of The SLBP Independent Mature MRNA	31 (199)	12.88	
34	12.31	Remodeling of Adherens Junctions	22 (121)	12.71	cadherin, the main adhesion molecule in adherens junctions, is tightly regulated by the UPS (41)
35	12.08	Cell Cycle Checkpoints	31 (202)	-	
36	11.92	Presenilin Action in Notch and Wnt Signaling	12 (46)	-	
37	11.5	Circadian Rythm Related Genes	31 (207)	13.68	
38	11.4	RNA Transport	27 (171)	14.03	
39	11.3	CLEC7A (Dectin-1) Signaling	24 (145)	11.77	
40	11.17	Formation of HIV Elongation Complex in The Absence of HIV Tat	28 (182)	-	
41	10.96	Cellular Senescence	55 (452)	14.27	
42	10.95	RNA Polymerase II Transcription Termination	15 (72)	-	
43	10.94	Calnexin/calreticulin Cycle	10 (36)	-	
44	10.86	Mechanisms of CFTR Activation By S-nitrosoglutathione (normal and CF)	11 (43)	9.96	
45	**10.75**	**Proteolysis Role of Parkin in The Ubiquitin-Proteasomal Pathway**	**15 (73)**	**15.85**	
46	10.75	Sterol Regulatory Element-Binding Proteins (SREBP) Signalling	15 (73)	-	
47	10.67	Metabolism	235 (2543)	10.35	
48	10.52	Cytoskeletal Signaling	40 (304)	12.9	
49	10.24	Brain-Derived Neurotrophic Factor (BDNF) Signaling Pathway	23 (144)	11.87	

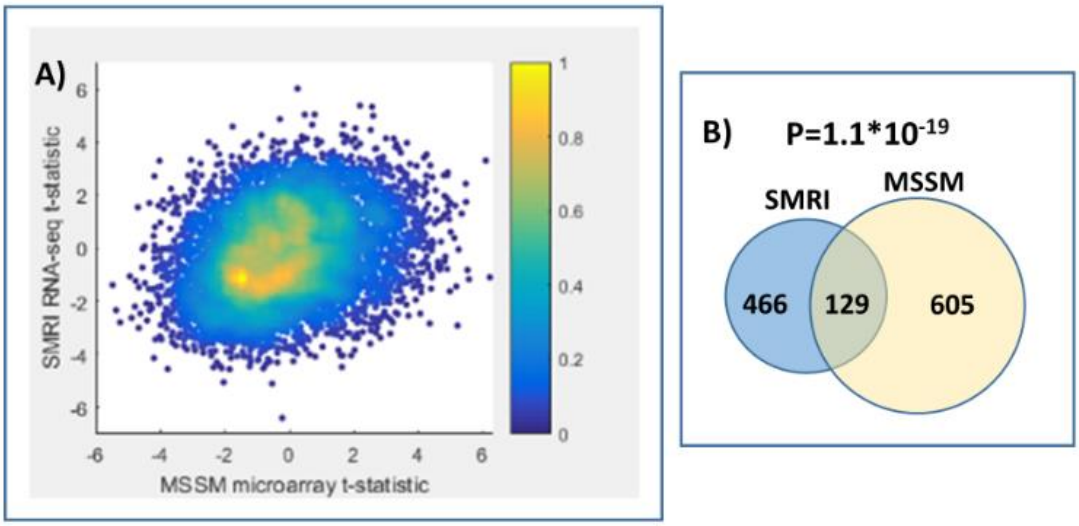

Figure 1. A) Binned density scatter plot comparing the t-statistics for case versus control differential expression between the independent MSSM replication cohort assayed on microarrays and the SMRI RNA-seq data; correlation between the statistics is 0.28 (P = 4.7*10−133). The colorbar represents the density in each cell, calculated by voronoi procedure (42) and normalized to values between 0 (minimal density) and 1 (maximal density). **B) Hypergeometric p-value calculation for the intersection between SMRI and MSSM down-regulated genes.** The 986 SMRI and 794 MSSM down-regulated genes were intersected with the 7,498 genes that are present in both cohorts, yielding 595 SMRI and 734 MSSM down-regulated genes, with 129 shared genes.

Pathway enrichment analysis was applied, and 27 and 48 pathways were enriched in up-regulated and down-regulated genes, respectively (results are listed in Tables 2S and 3S). Intersection between the SMRI 49 enriched pathways and the MSSM 48 enriched pathways in the down-regulated genes yields 30 pathways, listed in Table 2 (hypergeometric p-value: $2.5*10^{-36}$). Specifically, it can be seen that for four out of the five SMRI enriched UPS pathways, enrichment is replicated in the MSSM. A similar analysis of the up-regulated genes yields a hypergeometric p-value of $1.03*10^{-6}$.

3.3 A network view of the UPS identifies down-regulation of a tightly connected cluster of proteasome subunits

To further explore the UPS differential expression, we applied to the SMRI a differential expression network view (see methods). The network view included the genes of the Ubiquitin-Proteasome Dependent Proteolysis GeneAnalytics "superpathway" (31), which is representative of the UPS and was statistically significantly enriched in both SMRI and MSSM (see Table 2). It is important to note that the network view includes all 69 genes of the pathway for which network data from the STRING database (43) was available, and not only those 27 genes that were found to be down-regulated. As can be seen in Figure 2A, there is a cluster of tightly connected genes which are mostly down-regulated in schizophrenia (represented by bluish colours of the nodes). Interestingly, this cluster is composed of proteasome subunits, as shown in Figure 2B. The same analysis of the MSSM yields a similar view (Figure 5S).

3.4 Meta-analysis of SMRI and MSSM datasets identifies down-regulation of multiple proteasome subunits in STG samples of subjects with schizophrenia

To further explore the differential expression of the proteasome subunits genes, we performed a meta-analysis of the expression of each of the 39 proteasome subunit genes, whose expression has been measured by both SMRI and MSSM (see supplementary methods). The list of proteasome subunits genes, meta-analysis results and comparisons to previous gene expression and protein-level studies are described in Table 3. We detect for the first time down-regulation of multiple types of proteasome subunit genes. Overall, 12 out of 39 measured subunit genes were down-regulated (see Table 3).

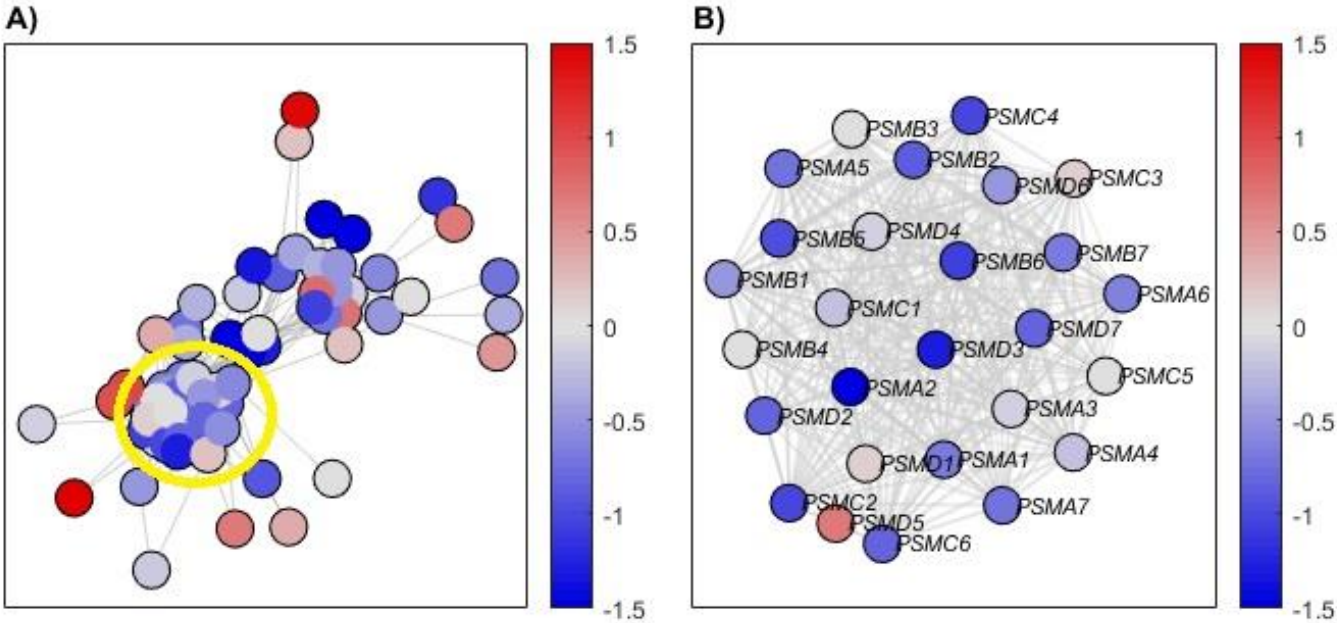

Figure 2. SMRI Differential expression network view: A) Ubiquitin-Proteasome Dependent Proteolysis superPathway. The node's colors correspond to the deviation of expression from the control samples group, in terms of standard deviation units (see Methods). The edges represent STRING database co-expression relations. Only genes that have co-expression relations with other genes in the network are displayed. A subgroup of highly-interconnected genes, coding for proteasome subunits, is circled **B) Zoom in on proteasome subunits.** The same plot as in A), for a subgroup of highly-interconnected genes coding for proteasome subunits (circled in A)).

Table 3. Proteasome subunits differential gene and protein level expression, in previous studies and in our meta-analysis. Previous gene expression studies' results were listed only for genes which were detected as differentially expressed in more than one study. Down-regulation findings are highlighted in blue. In the meta-analysis, a gene is defined as down-regulated if its summary measure is lower than zero and the confidence interval doesn't cross zero.

#	**Proteasome subunit genes**	**Previous gene expression studies**	**Previous protein level studies** (5, 11)	**Our meta-analysis (SMRI+MSSM)**	**SMRI+ MSSM meta-analysis summary measure [confidence interval]**
	Structural subunits				
	20S core α subunits				
1	PSMA1 (also named 20S α1)	Down-regulated in 2 studies (2, 16)	Not measured	unchanged	-0.37 [-0.97, 0.21]
2	PSMA2 (20S α2)		Not measured	Down-regulation	-1.13 [-1.68, 0.59]
3	PSMA3 (20S α3)		Not measured	unchanged	-0.63 [-1.67, 0.4]
4	PSMA4 (20S α4)		Not measured	unchanged	-0.43 [-0.9, 0.07]
5	PSMA5 (20S α5)		Not measured	Down-regulation	-0.61 [-1.13, -0.09]

6	PSMA6 (20S α6)		unchanged in (11)	Down-regulation	-0.63 [-1.15, -0.12]
7	PSMA7 (20S α7)		Not measured	Down-regulation	-0.79 [-1.32, 0.27]
	Catalytic subunits				
	20S core β subunits				
8	PSMB1 (20S β1)		Not measured	unchanged	-0.17 [-0.73, 0.37]
9	PSMB2 (20S β2)		Down-regulation trend (5) (p=0.08); unchanged in (11)	Down-regulation	-0.62 [-1.13, 0.11]
10	PSMB3 (20S β3)		Not measured	unchanged	-0.25 [-0.75, 0.25]
11	PSMB4 (20S β4)		Not measured	unchanged	-0.03 [-0.53, 0.46]
12	PSMB5 (20S β5)		unchanged (5, 11)	Down-regulation	-0.73 [-1.28, -0.18]
13	PSMB6 (20S β6)		Not measured	unchanged	-0.13 [-1.25, 0.97]
14	PSMB7 (20S β7)		Not measured	unchanged	-0.37 [-0.87, 0.13]
	Immunoproteasome β subunit genes				
	PSMB8 (20S β5i)		unchanged (5, 11)	unchanged in SMRI; absent in MSSM	
15	PSMB9 (20S β1i)		unchanged (5)	unchanged	-0.04 [-0.45, 0.54]
16	PSMB10 (20S β2i)		unchanged (5, 11)	unchanged	0.16 [-0..38, 0.71]
	Regulatory subunits				
	19S AAA-ATPase subunits (Rpt)				
17	PSMC1 (19S Rpt2)		unchanged in (5); Down-regulated in (11)	unchanged	0.02 [-0.48, 0.52]
18	PSMC2 (19S Rpt1)		Down-regulated in two studies (5, 11)	Down-regulation	-0.93 [-1.46, 0.4]
29	PSMC3 (19S Rpt5)		Unchanged in (5); Down-regulated in (11)	unchanged	-0.11 [-0.62, 0.38]
20	PSMC4 (19S Rpt3)		Down-regulated in two studies (5, 11)	Down-regulation	-0.67 [-1.19, 0.15]
21	PSMC5 (19S Rpt6)		Down-regulated in two studies (5, 11)	unchanged	0.02 [-0.48, 0.52]
22	PSMC6 (19S Rpt4)	Down-regulated in two studies (2, 4)	unchanged (5); Down-regulated in (11)	Down-regulation	-0.83 [-1.36, 0.3]
	19S non-ATPase subunits (Rpn)				
23	PSMD1 (19S Rpn2)		Not measured	unchanged	0.07 [-0.42, 0.58]
24	PSMD2 (19S Rpn1)		Not measured	unchanged	-0.03 [-1.49, 1.41]
25	PSMD3 (19S Rpn3)		Not measured	unchanged	-0.34 [-0.87, 0.18]
26	PSMD4 (19S Rpn10)		unchanged (5)	unchanged	0.09 [-0.41, 0.59]
27	PSMD5		Not measured	unchanged	0.17 [-0.69, 1.05]
28	PSMD6 (19S Rpn7)		Not measured	Down-regulation	-0.62 [-1.13, -0.1]
29	PSMD7 (19S Rpn8)		Not measured	unchanged	-0.07 [-1.02, 0.86]
30	PSMD8 (19S Rpn12)		Not measured	unchanged	-0.39 [-0.9, 0.11]
31	PSMD9 (19S Rpn4)		Not measured	unchanged	-0.39 [-1.42, 0.63]
32	PSMD10		Not measured	unchanged	0.13 [-0.36, 0.63]
33	PSMD11 (19S Rpn6)		unchanged (5)	Down-regulation	-0.73 [-1.25, -0.21]
34	PSMD12 (19S Rpn5)		Not measured	unchanged	-0.23 [-0.74, 0.26]

35	PSMD13 (19S Rpn9)		Not measured	unchanged	0.05 [-0.58, 0.69]
36	PSMD14 (19S Rpn11)		unchanged (5)	Down-regulation	-0.89 [-1.42, -0.37]
	11S subunits				
37	PSME1 (11S α)		Down-regulated in (5); unchanged in (11)	unchanged	-0.33 [-0.84, 0.16]
38	PSME2 (11S β)		unchanged (5, 11)	unchanged	0.29 [-0.65, 1.24]
39	PSME3 (11S gamma)		unchanged (5)	unchanged	-0.07 [-0.85, 0.7]

3.5 Down-regulation signal of multiple proteasome subunits in schizophrenia is replicated in 6 independent datasets of 5 different brain regions

In order to further validate our results and to check whether the down-regulation of the proteasome subunits in schizophrenia is specific to the STG or appears in additional regions, we repeated the differential expression network analysis of the 39 proteasome subunits genes using 6 additional schizophrenia vs. control independent datasets (fully described in the supplementary information): dorso-lateral prefrontal cortex (DLFPC) samples from Arion 2015 (4) and from Ramaker 2017 (44), STG samples from Barnes 2011 (45), Cerebellum samples from Chen 2018 (46), Brodmann area 23 (BA23) samples from the SMRI cohort, and Brodmann area 10 (BA10) samples from Mycox 2009 (47). The results are presented in Figure 3. The DLPFC samples of Arion 2015 (Figure 3A) exhibit pronounced down-regulation of most of the proteasome subunits, while in the DLPFC samples of Ramaker 2017 (Figure 3B), there is only slight tendency of down-regulation, though present in most of the genes; the binomial p-value for the number of genes with (even slightly) reduced expression versus the control group is $p= 6.4\ 10^{-6}$). Interestingly, while Ramaker 2017 (44) used brain samples composed of mixture of cells, Arion 2015 (4) used laser microdissection in order to capture pyramidal neurons of layers 3 and 5. Thus, the difference in the magnitude of the down-regulation might be due to dilution of the signal, caused by the mixture of cell types used in Ramaker 2017. The Cerebellum samples from Chen

2018 (46) (Figure 3D), BA10 samples from Mycox 2009 (Figure 3E) and BA23 SMRI (Figure 3F) samples show clear tendency for down-regulation (binomial p-values 6.9 10^{-7}, 1.2 10^{-5} and 0.04, respectively). However, the magnitude of down-

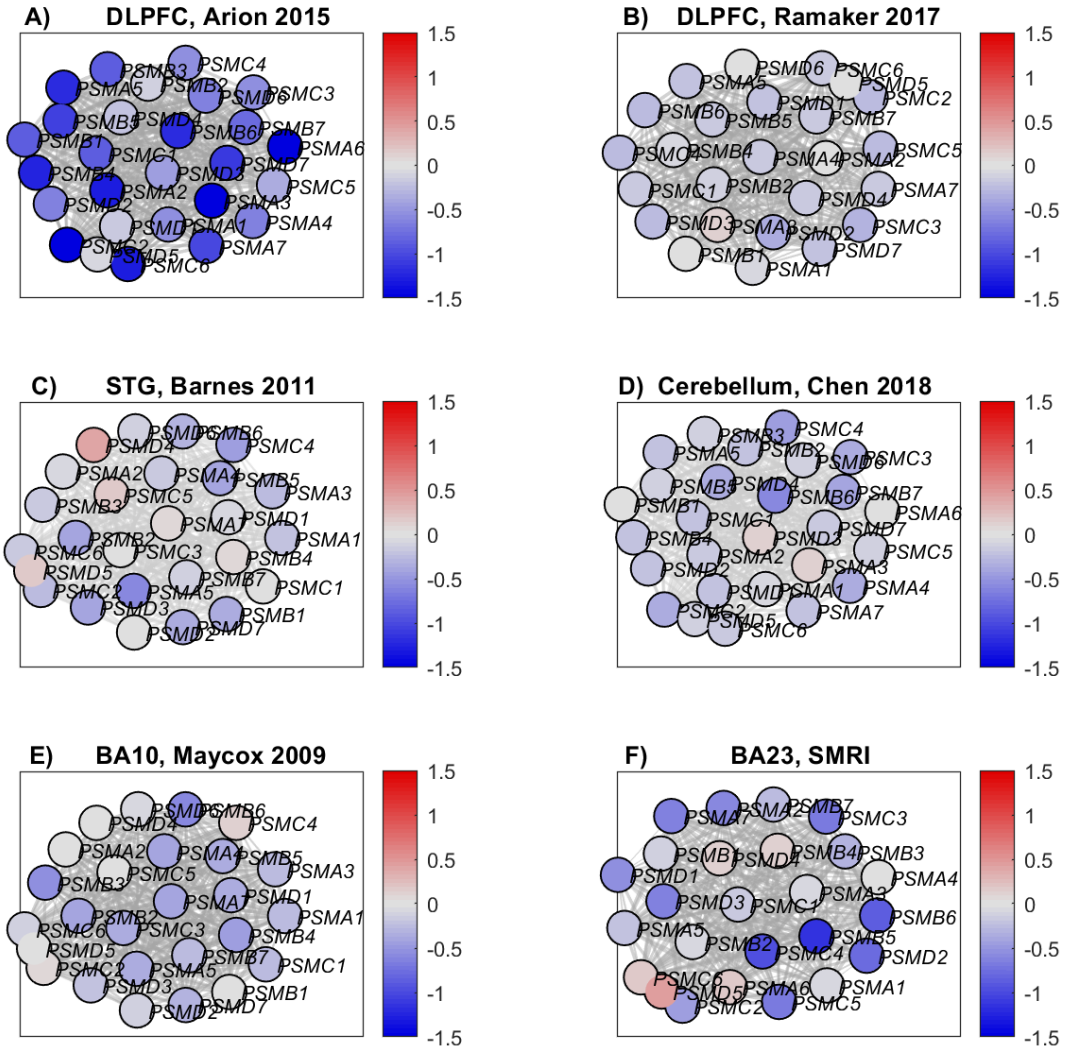

Figure 3. Proteasome subunits differential expression network view: The node's colors correspond to the deviation from the group of the control samples, in terms of standard deviation units (see Methods). The edges represent STRING database co-expression relations. Only genes that have co-expression relations with other genes in the network are displayed. **A) DLPFC, Arion 2015 dataset** (4)**. B) DLPFC, Ramaker 2017 dataset** (44)**. C) STG, Barnes 2011 dataset** (45). **D) Cerebellum, Chen 2018. E) BA10, Mycox 2009 dataset** (47) **F) BA23, SMRI dataset.**

regulation is modest (mostly less than 1 standard deviation). STG samples of Barnes 2011 (45) (Figure 3C) show a similar pattern. Down-regulation might be specific to neurons or even to subtypes of neurons; the fact that the brain samples in these datasets are composed of mixture of cells could dilute this signal. Overall, this analysis replicates the signal of down-regulation of multiple proteasome subunits, in both the STG and additional 4 brain regions, measured on 6 independent datasets.

4 Discussion

The main finding of our study is a global down-regulation of multiple proteasome subunits in post mortem brain samples of individuals with schizophrenia. This finding was replicated in 8 independent datasets of 5 different brain regions of overall 267 schizophrenia and 266 control samples, in both elderly and relatively young patients. A plausible scenario that stems from our results is that the observed down-regulation causes reduced proteasomal activity, which leads to the recently detected aggregation of ubiquitinated proteins in schizophrenia (10, 12).

As described in (12), the ubiquitinated proteins aggregates recently found in schizophrenia, were enriched with pathways related to nervous system development, suggesting that the aggregation of ubiquitinated proteins is related to disease pathogenesis, possibly through disruption of relevant pathways. While decreased proteasome activity is generally associated with ubiquitinated protein accumulation

(13), recent efforts to explore interruption of proteasome activity in schizophrenia led to inconsistent results (10, 11).

While our results of global down-regulation of proteasome subunits could suggest that dysfunction of the proteasome causes the observed accumulation of ubiquitinated proteins, this hypothesis is inconsistent with (10), where no change has been detected in proteasome activity between schizophrenia and controls, in both blood and orbitofrontal cortex samples. A possible explanation to this inconsistency is that the down-regulation signal we detect is specific to neurons or subtypes of neurons, and since the brain samples measured in (10) were composed of mixture of cell types, the signal was diluted.

Support for the hypothesis of proteasome dysfunction in schizophrenia comes from (11), where intra-cellular compartment-specific dysfunction of the proteasome activity in STG samples was found in patients with schizophrenia. However, It should be noted that no change in the protein levels of the structural subunit PSMA6 (also named 20S α6), or 4 catalytic subunits that were measured, were observed between schizophrenia and control samples (11). Based on that, it was concluded that there is no change in global proteasome content, which is inconsistent with our hypothesis. However, this conclusion might not be firmly based; only one structural subunit was measured, and in the same study six proteasome regulatory subunits were found to have statistically significant decreased protein levels in schizophrenia, while a subgroup showed also positive correlation with proteasome activity (11). In addition, while in (11) samples of 25 pair-matched schizophrenia and control subjects, originating from one dataset, were measured, we measure samples of more than 200 subjects with schizophrenia and 200

controls, of 8 independent datasets. In case the signal of global down-regulation of proteasome subunits we detect is indeed specific to subtypes of neurons, it could explain the inconsistency between this signal and the absent of change in one structural and 4 catalytic subunits protein levels in (11), in which samples composed of mixtures of cell types were studied.

The possibility that the down-regulation and dysfunction of the proteasome is specific to subtypes of the neurons is supported by our analysis of Arion 2015 dataset of laser microdissected neurons (4), where we detected higher magnitude of down-regulation signal compared to the other datasets, which are composed of mixture of cell types (see Figure 3). A dilution of the down-regulation signal when studying brain samples composed of mixture of cell types can explain why this signal hasn't been detected previously, although tens of relevant gene expression studies have been published. Actually, if we look at some of the datasets shown in Figure 3 separately (see for example, Figure 3B, C, D, E), it is hard to detect the down-regulation signal. Each single proteasome subunit is not pronouncedly down-regulated in schizophrenia, in those datasets. Only the combination of analysing the proteasome subunits as a group, with integrated analysis of independent datasets, enabled the detection of the global down-regulation signal.

This study, like other postmortem studies, is limited by several features. Every postmortem study represents only a snapshot of neurobiology at the end of life and cannot address the neurobiological abnormalities that may have existed when the disease was first expressed. This is especially important in the case of schizophrenia, as there is evidence that the pathogenesis of the disorder may be rooted in early

development (48). The fact that we compare independent cohorts of both relatively young and elderly subjects significantly strengthens the validity of the results, but doesn't fully overcome this serious limitation. The question of medication exposure must also be considered carefully, as exposure to antipsychotics has the potential to affect gene expression. This limitation was addressed by the correlation analysis we performed, which found no significant association between Fluphenazine equivalent dose and gene expression. In addition, the fact that the subjects of the cohorts significantly differ in their age suggests that duration of exposure to antipsychotics is unlikely to influence proteasome subunits gene expression substantively. The fact that the proteasome down-regulation signal we identified in two STG independent cohorts was replicated in 6 additional cohorts of different brain regions significantly increases the validity and generalizability of this signal. As gene expression do not always correlate with the levels of the proteins coded by the genes, the fact that we measure gene expression alone is a serious limitation, which causes difficulties in making conclusions regarding the biological consequences of the signal we detect. While few studies detected decreased protein levels of several proteasome subunits (5, 11), the results were not fully consistent and the recent studies of proteasome activity in schizophrenia were not consistent either (no change in activity was detected in (10), while (11) detected altered proteasome activity in schizophrenia). Thus, further study is needed in order to decipher the consequences of the global down-regulation of multiple proteasome subunits we detect in schizophrenia, in terms of protein levels and proteasome activity. In addition, there is need to study specific brain cell types (neurons, glial cells, etc.) separately, in order to detect whether the signal we detect is specific to a subgroup of the cells.

Overall, we detect, for the first time, global down-regulation of proteasome subunits expression in schizophrenia. This down-regulation might lead to proteasome dysfunction and be the cause of the aggregation of insoluble ubiquitinated proteins, which was recently found in a subset of brain samples of patients with schizophrenia. Further exploration of proteasome activity in schizophrenia and its relation to proteasome subunits expression and to the recently shown aggregation of insoluble ubiquitinated proteins could lead to a better understanding of the biological basis of the disease and might lead to improved nosology and to finding novel therapeutic targets.

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6 Disclosures

All authors declare that they have no conflict of interest.

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