Data Supplement for Nucifora et al., Increased Protein Insolubility in Brains From a Subset of Patients With Schizophrenia. Am J Psychiatry (doi: 10.1176/appi.ajp.2019.18070864)

Supplemental Tables

TABLE S1. Baseline demographic and population descriptors of brains (Harvard Brain Tissue Resource Center)

Characteristic	Mean (SD) or N (%)		p*
	Control (N=4)	SCZ (N=5)	
Age (mean years)	54.3 (2.9)	54.2 (3.5)	0.982
Sex (N)			0.524
Male	2 (50)	4 (80)	
Female	2 (50)	1 (20)	
PMI (mean hours)	25.0 (5.6)	21.9 (1.2)	0.252

*T-test if mean, Fisher's Exact if proportion.

TABLE S2A. Results by cluster for all brains (Harvard Brain Tissue Resource Center)

Characteristic	Positive[1] Vs. Negative Cluster		
	Mean (SD) or N (%)		
	Negative (N=7)	Positive (N=2)	p*
Group (N)			
Control	4 (57.1)	0	0.444
Schizophrenia	3 (42.9)	2 (100.0)	
Age (mean years)	53.9 (3.4)	55.5 (0.7)	0.536
Sex (N)			
Male	5 (71.4)	1 (50)	0.107
Female	2 (28.6)	1 (50)	
PMI (mean hours)	23.7 (4.4)	21.9 (0.1)	0.612

*T-test if mean, Fisher's Exact if proportion; 1. Clusters determined using hierarchical analysis

TABLE S2B. Results by cluster for brains from only schizophrenia patients (Harvard Brain Tissue Resource Center)

Characteristic	Positive[1] Vs. Negative Cluster		
	Mean (SD) or N (%)		
	Cluster 1 (Negative) (N=3)	Cluster 2 (Positive) (N=2)	p*
Age (mean years)	53.3 (4.6)	55.5 (0.7)	0.576
Sex (N)			
Male	3 (100.0)	1 (50)	0.400
Female	0	1 (50)	
PMI (mean hours)	21.8 (1.8)	21.9 (0.1)	0.952

*T-test if mean, Fisher's Exact if proportion; 1. Clusters determined using hierarchical analysis

TABLE S3. Baseline demographic and population descriptors of brains (University of Pittsburgh Brain Bank)

Characteristic		Mean (SD) or N (%)		p*
		Control (N=19)	SCZ (N=19)	
Age (mean years)		45.6 (10.9)	48.7 (12.7)	0.417
Sex (N)				1.000
	Male	14 (73.7)	14 (73.7)	
	Female	5 (26.3)	5 (26.3)	
Race (N)				0.476
	White	14 (73.7)	12 (63.2)	
	Black	4 (21.0)	7 (36.8)	
	Other	1 (5.3)	0	
Medication Use (N)				
	Any	10 (52.6)	18 (94.7)	0.008
	Antipsychotic	0	16 (84.2)	<0.001
	Anticonvulsant	0	5 (26.3)	0.046
	Antidepressant	0	8 (42.1)	0.003
	Benzodiazepine	0	2 (10.5)	0.486
	Other	10	13 (68.4)	0.508
PMI (mean hours)		17.6 (6.7)	18.2 (7.7)	0.784
RIN (mean)		8.3 (0.4)	8.1 (0.6)	0.288
Brain pH (mean)		6.7 (0.2)	6.5 (0.3)	0.006
Storage (mean months)		153.4 (44.9)	145.5 (37.5)	0.561

*T-test if mean, Fisher's Exact if proportion

TABLE S4. Baseline demographic and population descriptors of brains (University of Texas Southwestern Medical Center)

Characteristic		Mean (SD) or N (%)		
		Control (N=18)	SCZ (N=18)	p*
Age (mean years)		51 (14.7)	50.7 (15.2)	0.956
Sex (N)				
	Male	12 (66.7)	11 (61.1)	1.000
	Female	6 (33.3)	7 (38.9)	
Race (N)				
	White	16 (88.9)	13 (72.2)	0.308
	Black	0	3 (16.7)	
	Other	2 (11.1)	2 (11.1)	
Medications Use (N)				
	Any	10 (55.6)	15 (83.3)	0.146
	Antipsychotic	0	13 (72.2)	<0.001
	Anticonvulsant	0	5 (27.8)	0.045
	Antidepressant	0	8 (44.4)	0.003
	Benzodiazepine	0	5 (27.8)	0.045
	Other	10 (55.6)	11 (61.1)	1.000
PMI (mean hours)		19.0 (6.7)	18.9 (8.4)	0.991
RIN (mean)		8.5 (0.9)	8.7 (1.1)	0.601
Brain pH (mean)		6.5 (0.4)	6.6 (0.3)	0.533
Storage (mean months)		143.8 (19.5)	152.0 (22.4)	0.246

*T-test if mean, Fisher's Exact if proportion

TABLE S5. Baseline demographic and population descriptors of brains from all three studies combined (Harvard Brain Tissue Resource Center, University of Pittsburgh Brain Bank, and University of Texas Southwestern Medical Center)

Characteristic	Mean (SD) or N (%)		p*
	Control (N=37, ^N=41)	SCZ (N=37, ^N=42)	
Age (mean years^)	48.8 (12.5)	50.2 (13.1)	0.611
Sex (N^)			
Male	28 (68.3)	29 (69.1)	1.00
Female	13 (31.7)	13 (30.9)	
Race (N)			
White	30 (81.1)	25 (67.6)	0.211
Black	4 (10.8)	10 (27.0)	
Other	3 (8.1)	2 (5.4)	
Medication Use (N)			
Any	20 (54.1)	33 (89.2)	0.002
Antipsychotic	0	29 (78.4)	<0.001
Anticonvulsant	0	10 (27.0)	0.001
Antidepressant	0	16 (43.2)	<0.001
Benzodiazepine	0	7 (18.9)	0.011
Other	20 (54.1)	24 (64.9)	0.478
PMI (mean hours^)	18.9 (6.8)	19.0 (7.5)	0.975
RIN, mean	8.4 (0.7)	8.4 (0.9)	0.944
Brain pH (mean)	6.6 (0.3)	6.5 (0.3)	0.211
Storage (mean months)	148.7 (34.8)	148.7 (30.8)	0.998

^Larger Ns were available for data that were available from all three data sets.; *T-test if mean, Fisher's Exact if proportion

TABLE S6. Mass spectrometry quantification: complete data set.

(See the Excel spreadsheet in a separate supplemental file.)

TABLE S7. Identification of proteins significantly enriched at several stages of the ubiquitination process in the insoluble fraction, from brains with increased protein insolubility and ubiquitination compared to those without.

Activation	FXBO3, UBA1, UBA6, STUB1
Conjugation	HUWE1, TRIM36, UBE2D3, ATG3, LRSAM1
Proteolysis	PSMC2, PSMC5, PSMD12
Deconjugation	OTUB1, USP9X, USP11, USP14, UCHL1, UCHL5

TABLE S8. Results from cell type enrichment analysis

RNA-Seq Data Set	Cell Type	SD	p	FDR
Darmanis	Neurons	10.62	<0.0001	<0.0001
Darmanis	Oligodendrocytes	2.10	0.021	0.062
Darmanis	OPCs	–6.14	1.000	1.000
Darmanis	Astrocytes	–0.07	0.519	1.000
Darmanis	Microglia	–4.95	1.000	1.000
Darmanis	Endothelial cells	–0.66	0.740	1.000
Lake	In1	–2.14	0.986	1.000
Lake	In2	0.01	0.486	1.000
Lake	In3	–2.93	0.999	1.000
Lake	In4	1.64	0.052	0.139
Lake	In5	–1.06	0.858	1.000
Lake	In6	2.82	0.005	0.015
Lake	In7	–3.22	1.000	1.000
Lake	In8	–0.53	0.700	1.000
Lake	Ex1	2.96	0.002	0.009
Lake	Ex2	–0.59	0.715	1.000
Lake	Ex3	6.11	<0.0001	<0.0001
Lake	Ex4	4.03	<0.0001	<0.0001
Lake	Ex5	2.94	0.002	0.009
Lake	Ex6	–2.37	0.993	1.000
Lake	Ex7	–0.23	0.583	1.000
Lake	Ex8	–1.24	0.894	1.000

Supplemental Figures

FIGURE S1, A–E. Cold sarkosyl fractionation was used to obtain insoluble protein fractions from five schizophrenia patients and four controls from the Harvard Brain Tissue Resource Center for our pilot study. (A) When insolubility and ubiquitination were jointly analyzed, there was a group of patients with schizophrenia that separated from all other patients and controls. Controls are in blue and patients are in red. (B) Hierarchical clustering analysis determined that two of the schizophrenia patients (S41 and S42, Cluster 2) clustered separately from all other patients and controls. (C) Patients in Cluster 2 have significantly more protein insolubility than the patients and controls in Cluster 1 ($p<0.001$). (D) The patients in Cluster 2 were also significantly different than the patients and controls in Cluster 1 based on the amount of ubiquitination in the insoluble fraction ($p<0.001$). Blue represents controls with low levels of the markers, pink indicates patients with low levels of the markers, and red indicates patients with high levels of the markers, based on hierarchical cluster analysis. (E) The ubiquitination was strikingly higher in the subset of patient brains from Cluster 2 than in the brains of the other patients or of controls in Cluster 1.

FIGURE S1A: Comparison of Protein Insolubility to Ubiquitin Reactivity (Harvard Brain Tissue Resource Center)

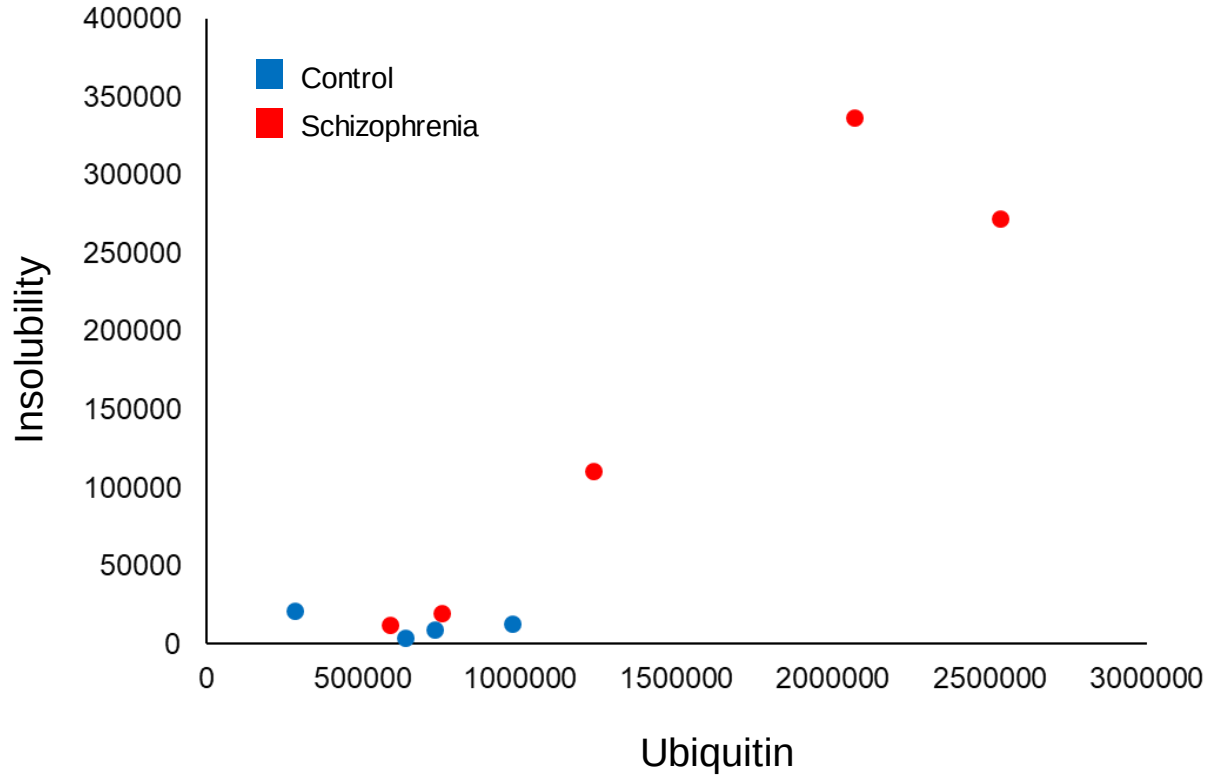


FIGURE S1B: Hierarchical Cluster Analysis (Harvard Brain Tissue Resource Center)

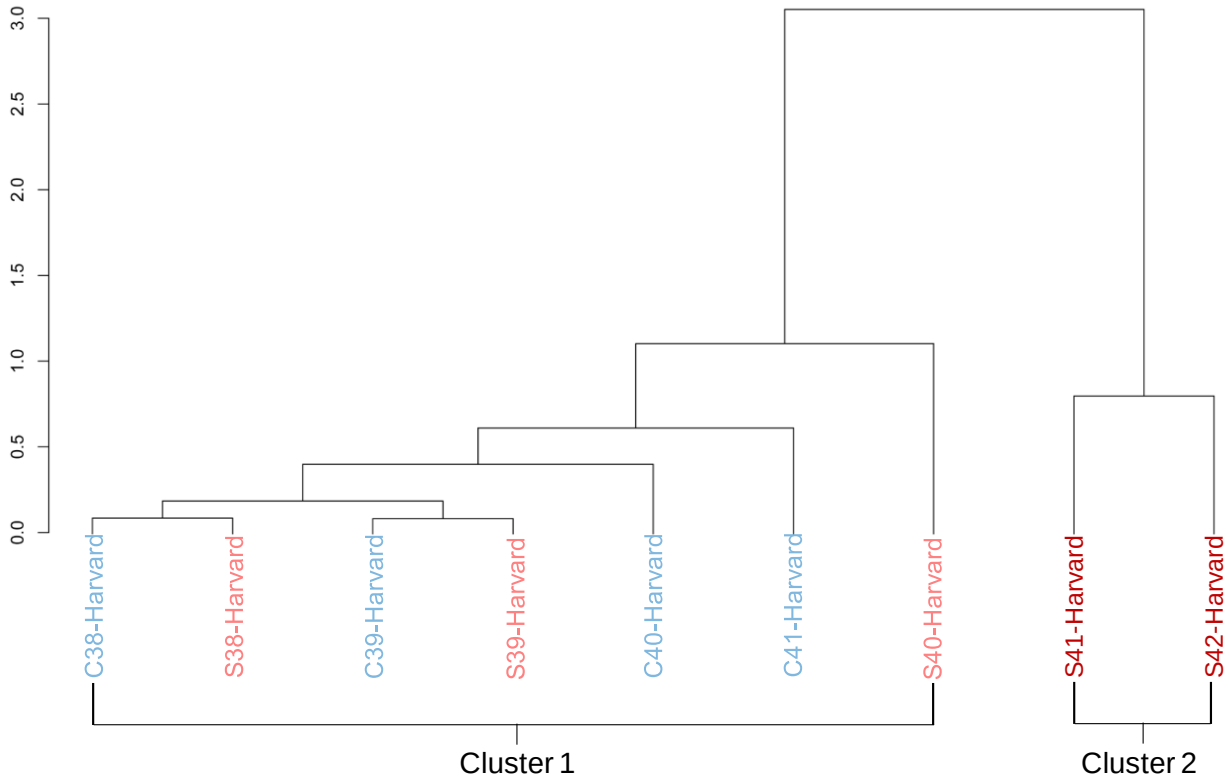
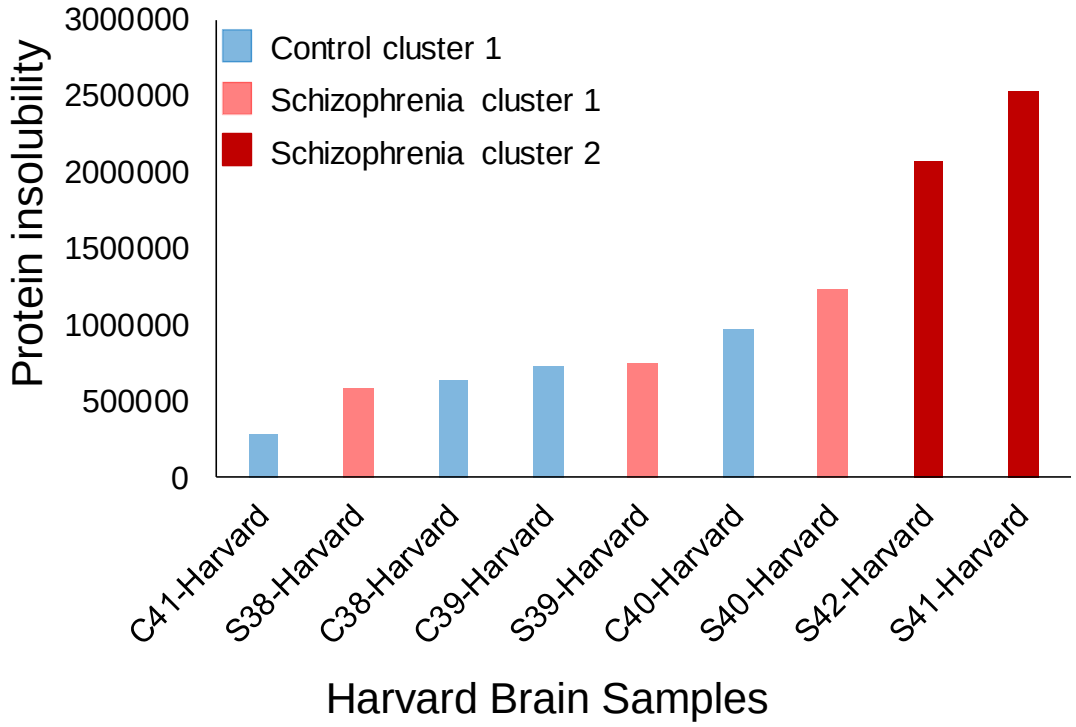


FIGURE S1C and D: Increased Ratio of Insoluble to Total Protein and Ubiquitination in a Subset of Schizophrenia Brains Compared with Controls (Harvard Brain Tissue Resource Center)

C



D

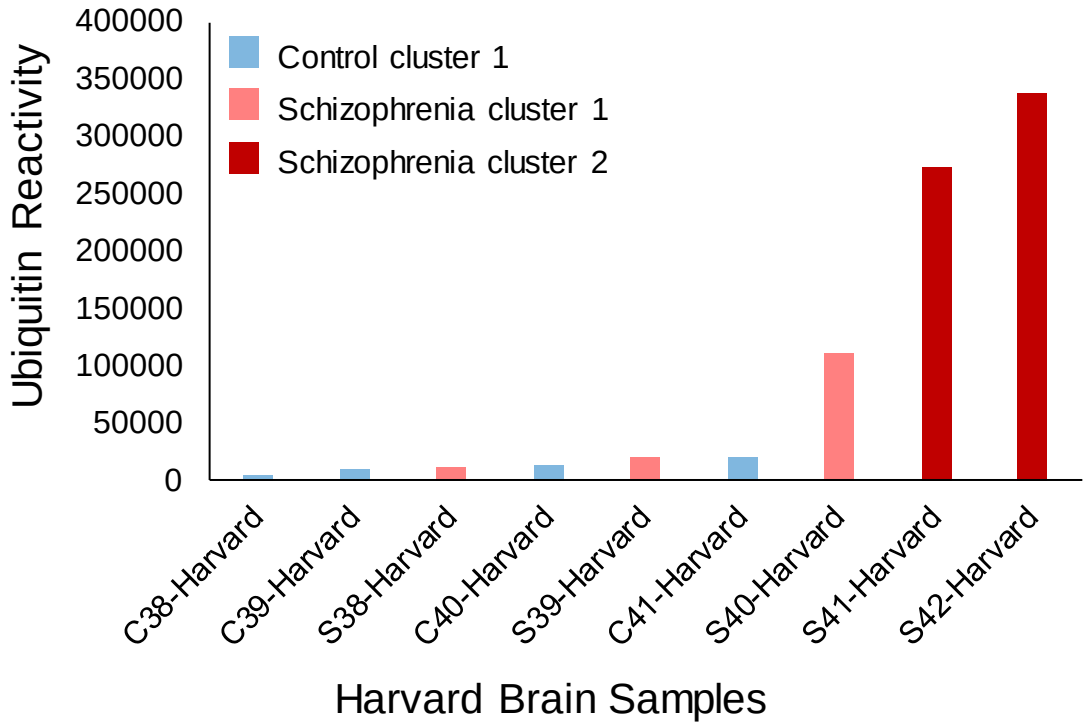

400000
350000
300000
250000
200000
150000
100000
50000
0
Ubiquitin Reactivity
Control cluster 1
Schizophrenia cluster 1
Schizophrenia cluster 2
C38-Harvard
C39-Harvard
S38-Harvard
C40-Harvard
S39-Harvard
C41-Harvard
S40-Harvard
S41-Harvard
S42-Harvard
Harvard Brain Samples

FIGURE S1E. Ubiquitination Western Blot (Harvard Brain Tissue Resource Center)

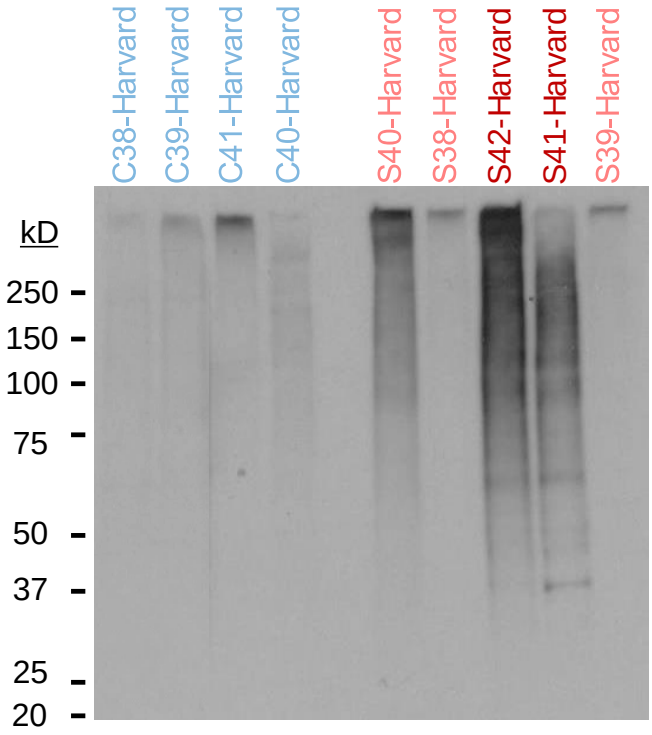


FIGURE S2. The total amount of starting material was not significantly different when the mean of three representative patients with increased protein insolubility was compared to the mean of three representative controls. The data detect an overall shift in the amount of protein from the soluble fraction to the insoluble fraction, with the patients having significantly more protein in the insoluble fraction and significantly less protein in the soluble fraction compared to the controls. When the soluble and insoluble fractions values were combined, they added back to approximately the same amount of total starting material. **denotes p<0.001, NS denotes not statistically significant.

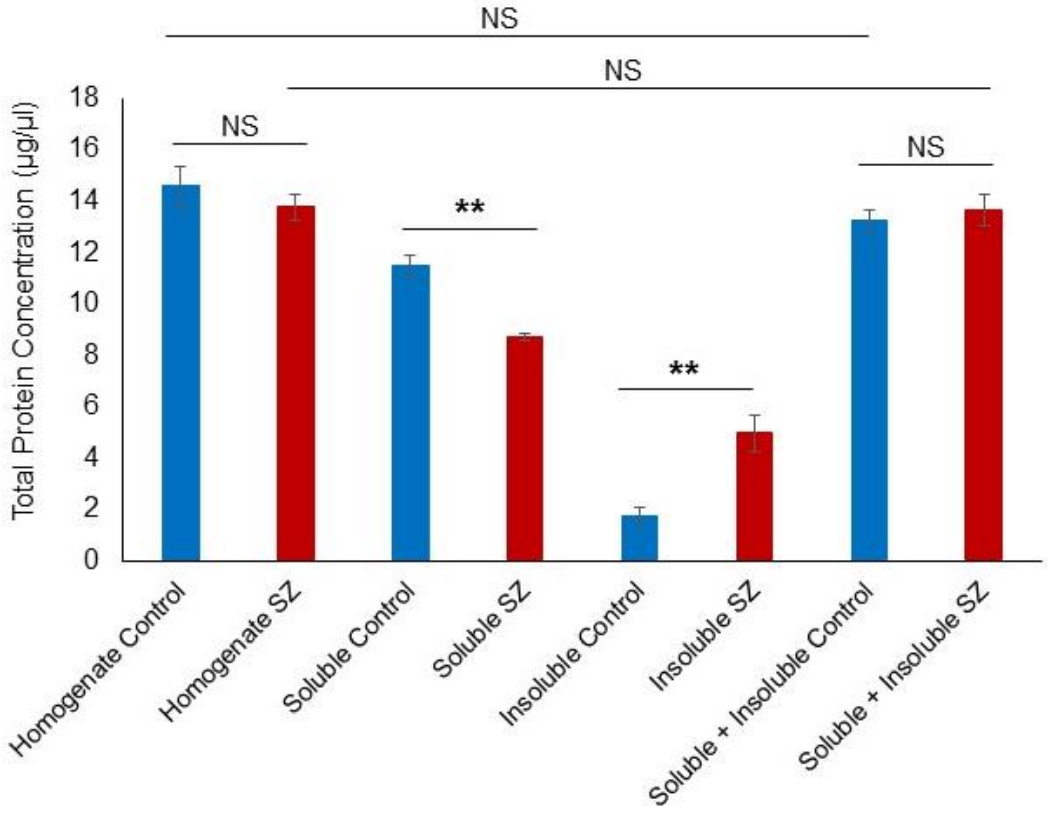


FIGURE S3. Protein insolubility and ubiquitination are not caused by antipsychotics. We performed the sarkosyl fractionation protocol on brains from rats that were subjected to 4.5 months of oral haloperidol, risperidone or water. (A) There was not a significant effect on the amount of protein insolubility for the three conditions by one-way ANOVA (p=0.961). (B) There was not a significant effect on the amount of ubiquitination for the three conditions by one-way ANOVA (p=0.598). NS denotes not statistically significant.

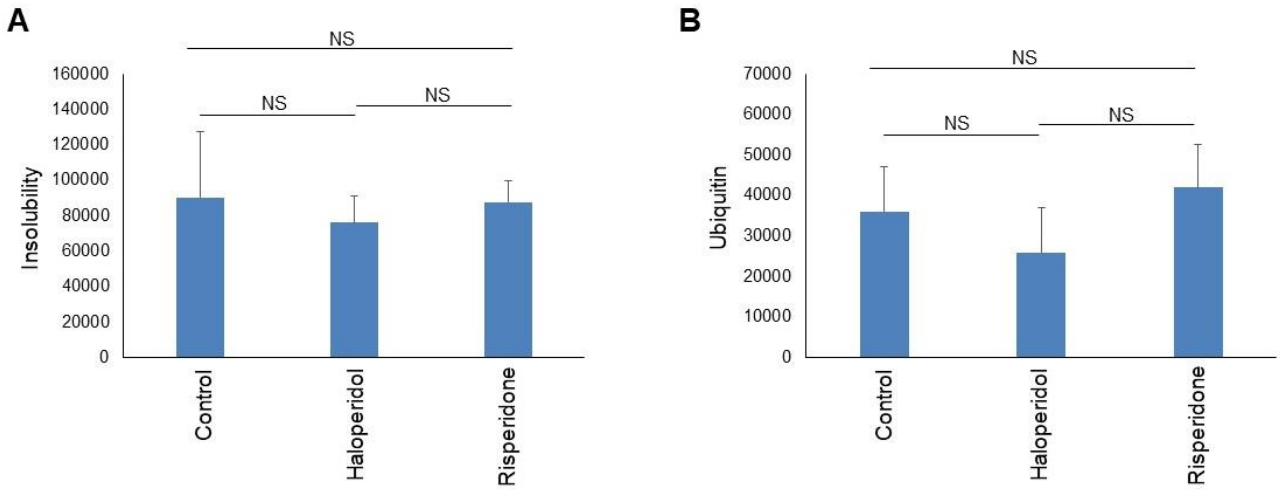


FIGURE S4. Biological processes and pathways associated with protein insolubility. (A) Gene set enrichment analysis identified axon target recognition as the Gene Ontology (GO) biological process most enriched for insoluble proteins. (B) Pathway analysis revealed the most significant enrichment for insoluble proteins in neurological disease for the disease and disorder category, cell assembly and organization for the molecular and cellular function category, and nervous system development and function for the physiological system development and function category.

A. Gene Ontology Enrichment

GO biological process	#	#	expected	Fold enrich-ment	+/-	raw p-value	FDR
Axon target recognition	3	2	0.02	82.52	+	6.28E-04	2.40E-02
└Cellular process	15437	157	124.72	1.26	+	7.27E-10	5.67E-07
└Axonogenesis	372	11	3.01	3.66	+	2.75E-04	1.27E-02
└Axon development	403	11	3.26	3.38	+	5.29E-04	2.12E-02
└Neuron projection development	663	17	5.36	3.17	+	3.47E-05	2.52E-03
└Plasma membrane bounded cell projection organization	1097	28	8.86	3.16	+	8.67E-08	2.51E-05
└Cell projection organization	1132	28	9.15	3.06	+	1.63E-07	3.69E-05
└Cellular component organization	5565	83	44.96	1.85	+	7.63E-10	5.67E-07
└ Cellular component organization or biogenesis	5795	86	46.82	1.84	+	2.53E-10	2.20E-07
└Neuron development 21	807	18	6.52	2.76	+	1.17E-04	6.56E-03
└Cell development 26	1572	30	12.70	2.36	+	1.22E-05	1.02E-03
└Cell differentiation 32	3610	53	29.17	1.82	+	8.63E-06	7.61E-04
└ Cellular developmental process 37	3698	54	29.88	1.81	+	6.98E-06	6.72E-04
└ Developmental process 44	5618	75	45.39	1.65	+	1.28E-06	1.78E-04
└Anatomical structure development 32	5253	71	42.44	1.67	+	1.88E-06	2.31E-04
└Neuron differentiation 26	992	22	8.01	2.75	+	2.11E-05	1.67E-03
└Generation of neurons	1473	34	11.90	2.86	+	3.19E-08	1.16E-05
└Neurogenesis	1572	36	12.70	2.83	+	1.40E-08	6.06E-06
└Nervous system development	2287	50	18.48	2.71	+	4.16E-11	4.32E-08
└System development	4272	65	34.51	1.88	+	8.76E-08	2.49E-05
└Multicellular organism development	4888	68	39.49	1.72	+	1.56E-06	2.02E-04
└Multicellular organismal process	6790	83	54.86	1.51	+	1.06E-05	9.10E-04
└Neuron projection morphogenesis	487	12	3.93	3.05	+	7.18E-04	2.65E-02
└Plasma membrane bounded cell projection morphogenesis	491	12	3.97	3.03	+	7.69E-04	2.82E-02
└Cell projection morphogenesis	494	12	3.99	3.01	+	8.10E-04	2.92E-02
└Cellular component morphogenesis	791	16	6.39	2.50	+	7.98E-04	2.90E-02
└Cell part morphogenesis	514	13	4.15	3.13	+	3.40E-04	1.49E-02
└Cell morphogenesis involved in neuron differentiation	433	11	3.50	3.14	+	9.39E-04	3.22E-02
└Cell morphogenesis involved in differentiation	533	13	4.31	3.02	+	4.75E-04	1.94E-02

B. Ingenuity Pathway Analysis

Diseases and Disorders	p-value range	# molecules
Neurological disease	3.82E-09 ~ 7.56E-49	457
Cancer	4.10E-09 ~ 1.11E-47	847
Organismal injury and abnormalities	4.10E-09 ~ 1.11E-47	856
Psychological disorders	4.53E-09 ~ 3.83E-47	267
Gastrointestinal disease	3.18E-09 ~ 1.51E-44	798
Molecular and Cellular Functions	**p-value range**	**# molecules**
Cellular assembly and organization	4.61E-09 ~ 1.73E-75	398
Cellular function and maintenance	4.61E-09 ~ 1.73E-75	448
Cell morphology	3.46E-09 ~ 4.65E-62	324
Cellular development	6.08E-09 ~ 4.65E-62	326
Cellular growth and proliferation	6.08E-09 ~ 4.65E-62	325
Physiological System Development and Function	**p-value range**	**# molecules**
Nervous system development and function	4.55E-09 ~ 4.65E-62	365
Organismal development	3.90E-09 ~ 4.65E-62	348
Tissue development	4.61E-09 ~ 4.65E-62	298
Tissue morphology	2.29E-09 ~ 1.77E-48	257
Embryonic development	3.90E-09 ~ 8.47E-42	224

FIGURE S5. Cell type enrichment of insoluble proteins. Using the Darmanis et al. RNA-seq dataset, insoluble proteins were found to be significantly enriched in neurons. Neuron subtype analysis, using the Lake et al. RNA-seq dataset, revealed significant enrichment of insoluble proteins in one inhibitory subtype (In6) and four excitatory subtypes (Ex1, Ex3, Ex4, and Ex5). Insoluble proteins were not significantly enriched in oligodendrocytes, oligodendrocyte progenitor cells (OPC), astrocytes, microglia, or endothelial cells. * Significant (FDR<0.05); SD=Standard deviations from the bootstrapped mean (N=20,000 bootstraps); In=Inhibitory neurons; Ex=Excitatory neurons.