SUPPORTING INFORMATION

An Optimised Method for the Proteomic Analysis of Low Volumes of Cell Culture Media and the Secretome: The Application and the Demonstration of Altered Protein Expression in iPSC-Derived Neuronal Cell Lines from Parkinson's Disease Patients.

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Figure S-2. Representative confocal micrograph of iPSC-derived neurons from control and from a patient with a triplication of the *SNCA* gene which codes for the protein α-synuclein. To confirm neuronal differentiation iPSC-derived neurons were stained for neuronal markers β III tubulin (TuJ1) and tyrosine hydroxlase (TH, a) or α-synuclein (b).

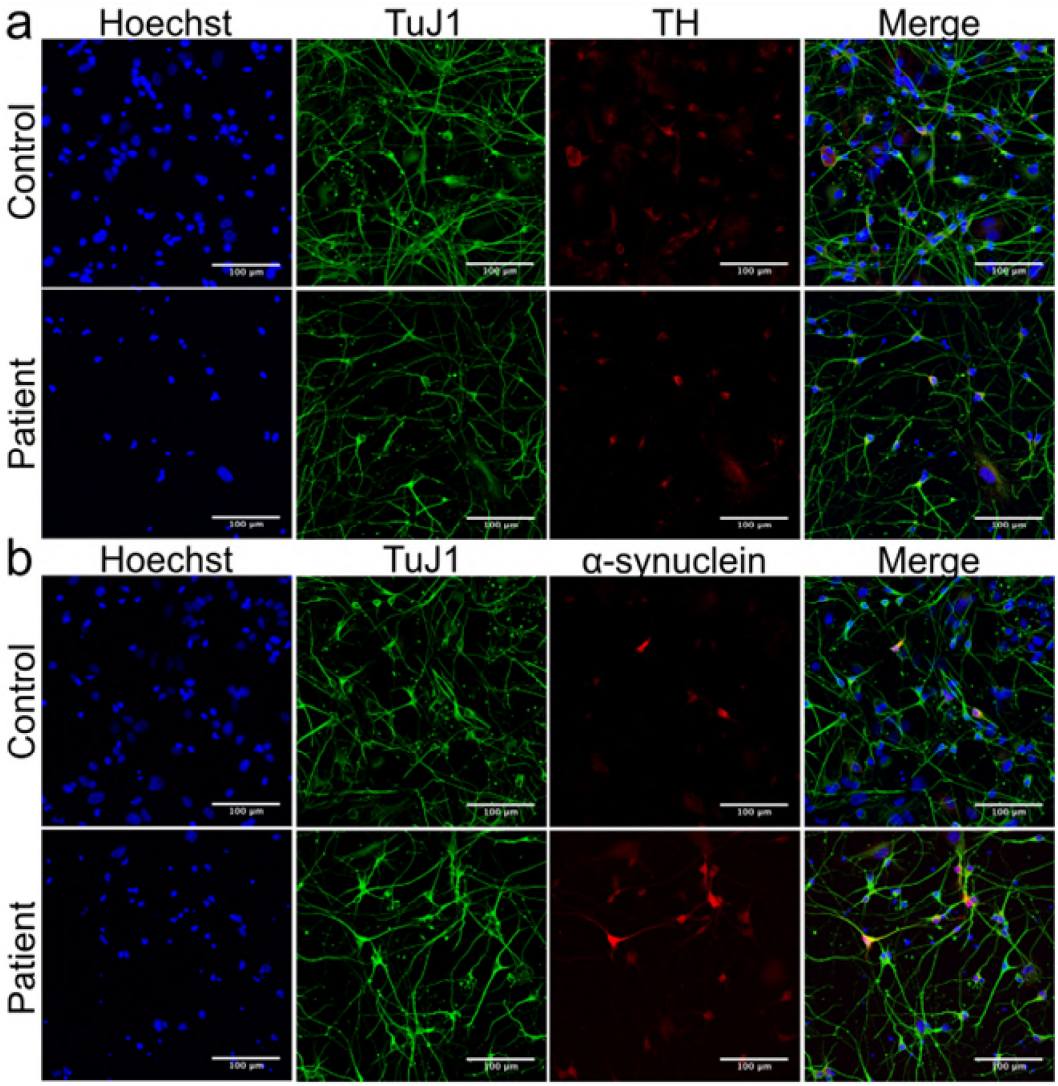

Figure S-2. Venn diagram showing proteins identified in standard cell media using different preparation techniques.

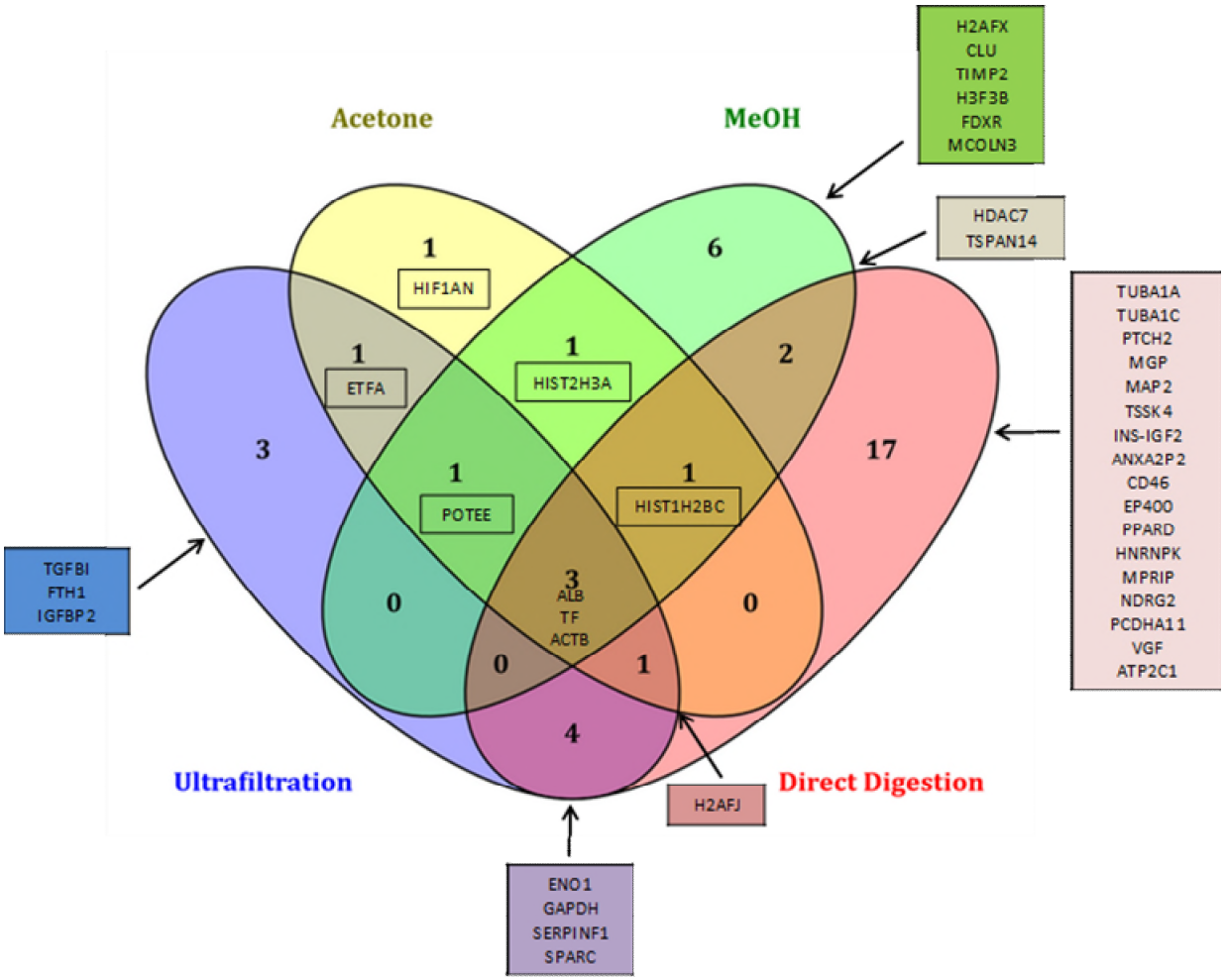

Figure S-3. Effect of B27 removal on cell viability. Cell viability measured using CellTitre-Glo in cells incubated for 24 hours in B-27 free media compared with those in regular media (basal). P<0.05 t-test, n=3 independent experiments. Dots represent data from each well, lines represent mean + SEM, data normalized to basal for each experiment.

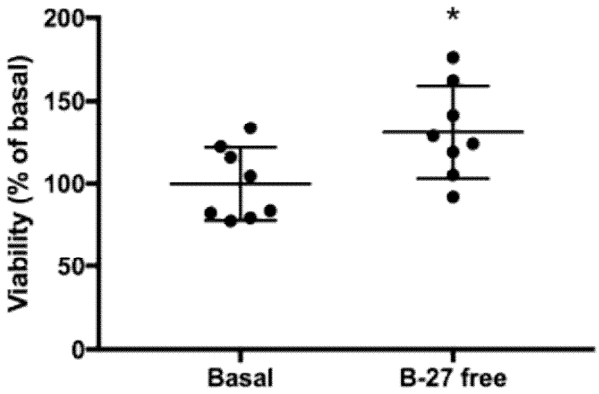

Figure S-4. Functional classification of identified proteins in cell supernatant conditioned for 3, 6 and 24h with iPSC-derived cell supernatant, using Panther software. Proteins annotated as belonging to extracellular matrix and extracellular region are highlighted in green.

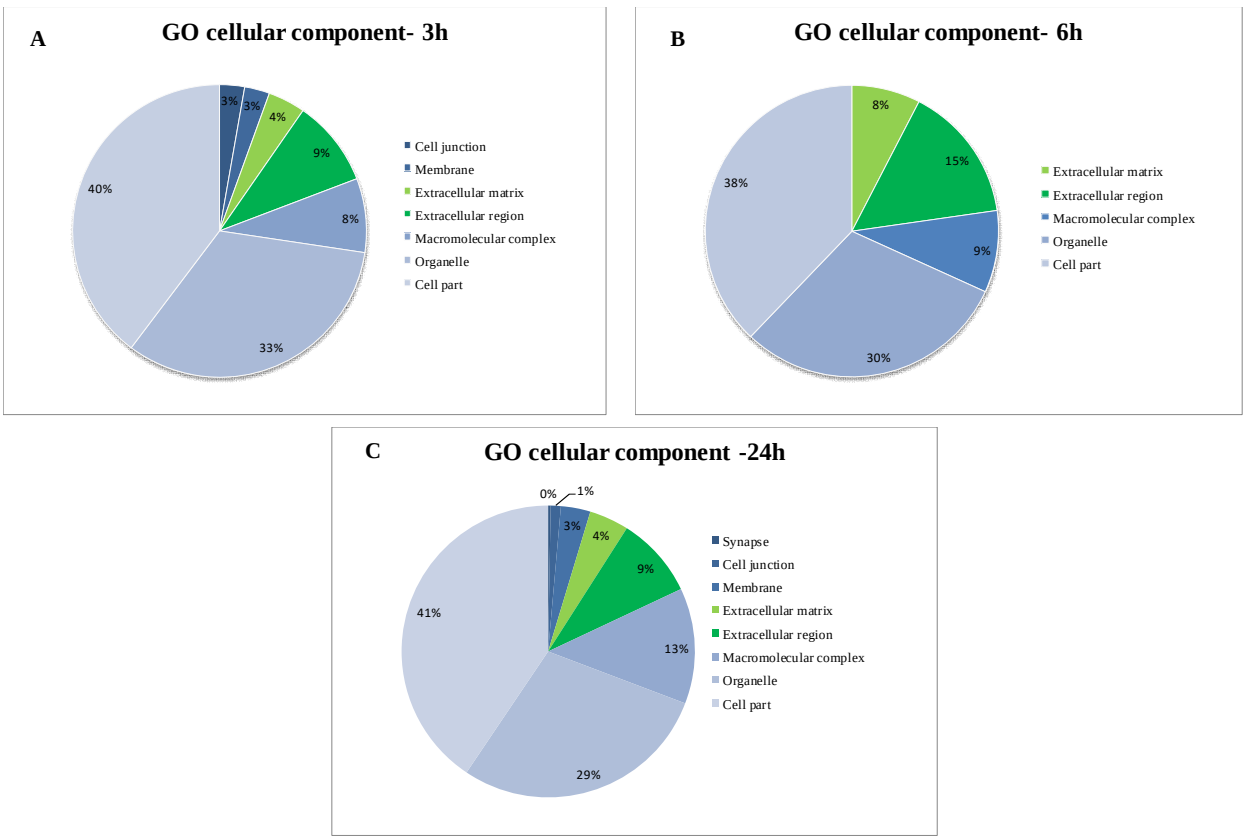

Figure S-5. Gene ontology analysis of B27-free supernatant conditioned for 3h with iPSC-derived neurons, using the Web-based Gene Set Analysis Toolkit (WebGestalt). Proteins annotated as belonging to the extracellular space or extracellular matrix are given, and extracellular proteins annotated also by Panther software are highlighted in blue.

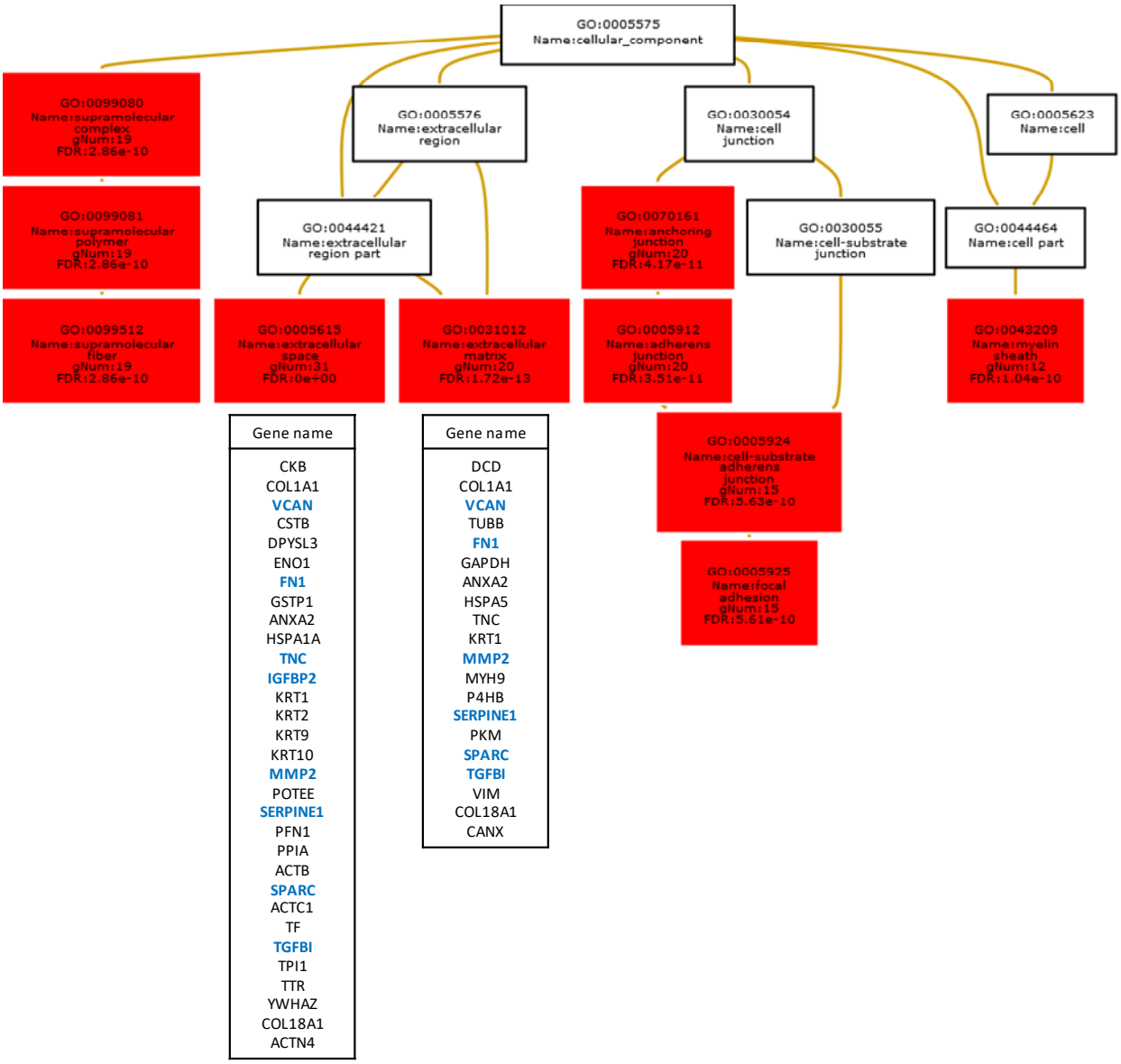

Figure S-6. Gene ontology analysis of B27-free supernatant conditioned for 6h with iPSC-derived neurons, using the Web-based Gene Set Analysis Toolkit (WebGestalt). Proteins annotated as belonging to the extracellular space or extracellular matrix are given, and extracellular proteins annotated also by Panther software are highlighted in blue.

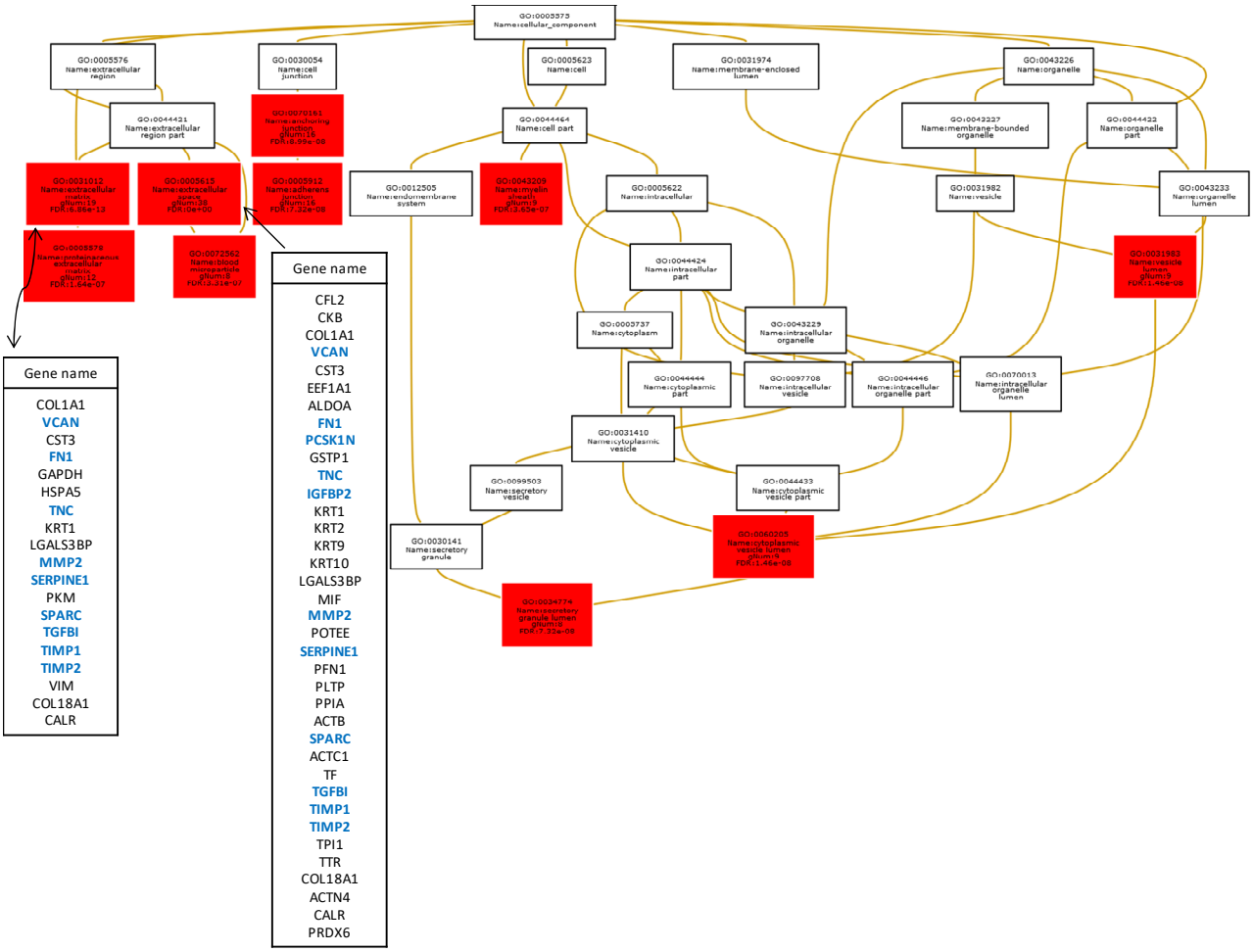

Figure S-7. Gene ontology analysis of pathways upregulated in the secretome of control cell line (A) and cell line with triplication in the SNCA gene (B). Proteins annotated as belonging to particular pathways are given.

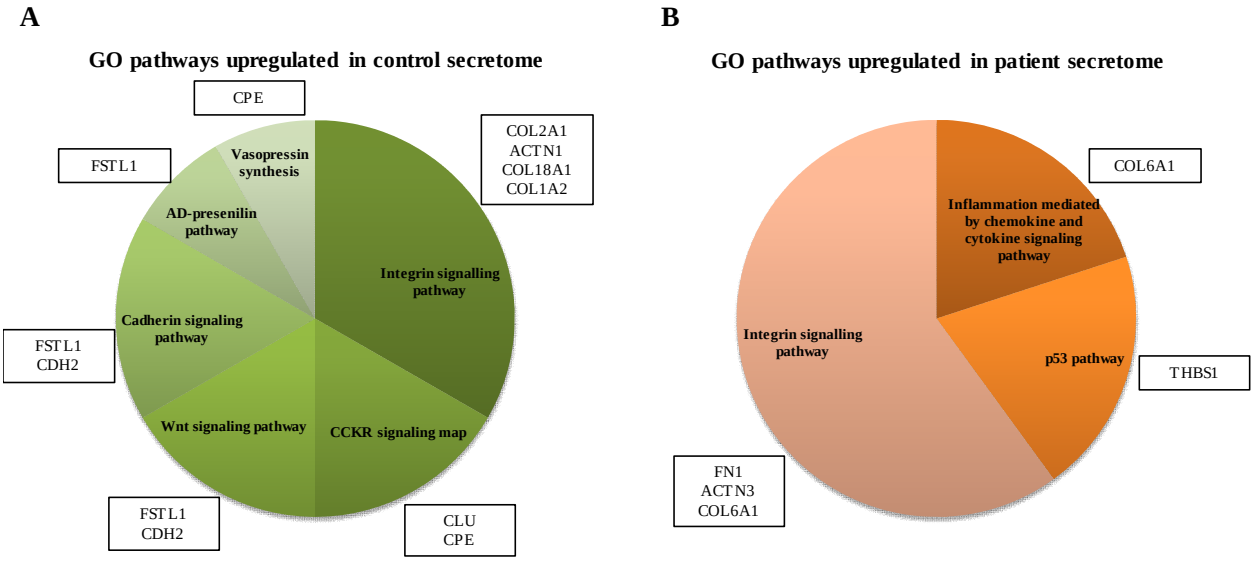

Figure S-8. A representative Baseline Peak Ion Chromatogram of B27-free cell supernatant precipitated in 10% TCA in acetone prior digestion.

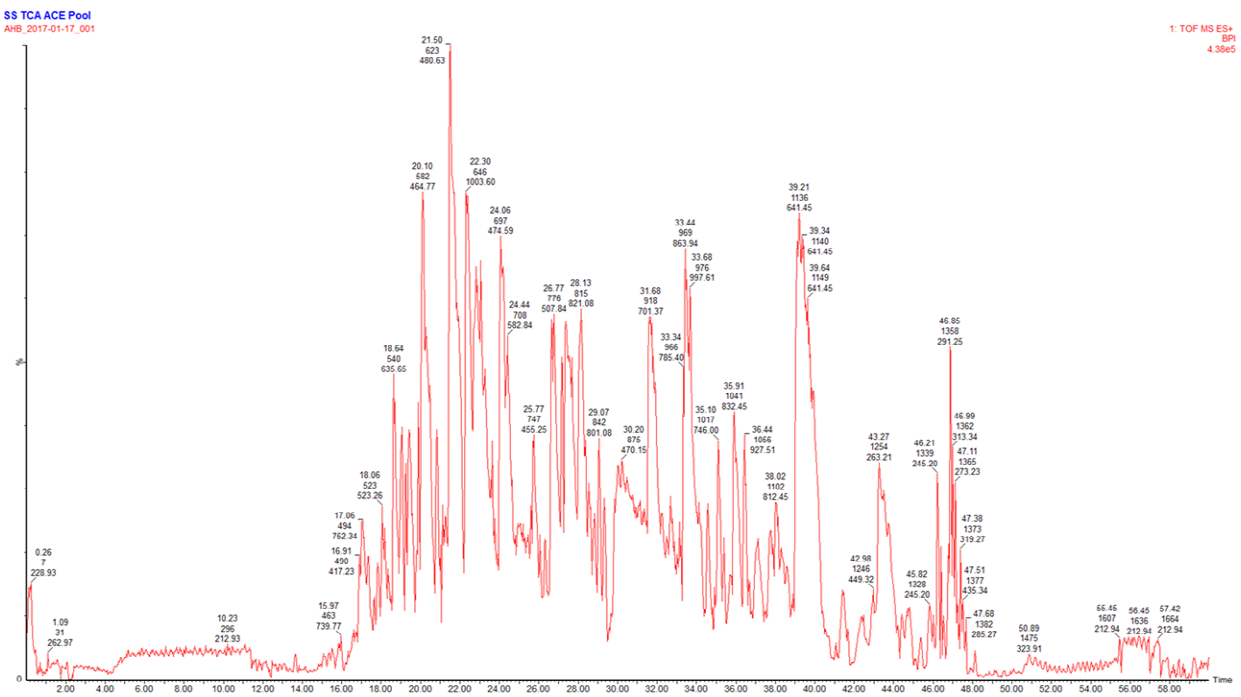

Figure S-9. A representative Baseline Peak Ion Chromatogram of B27-free cell supernatant precipitated in acetone prior digestion.

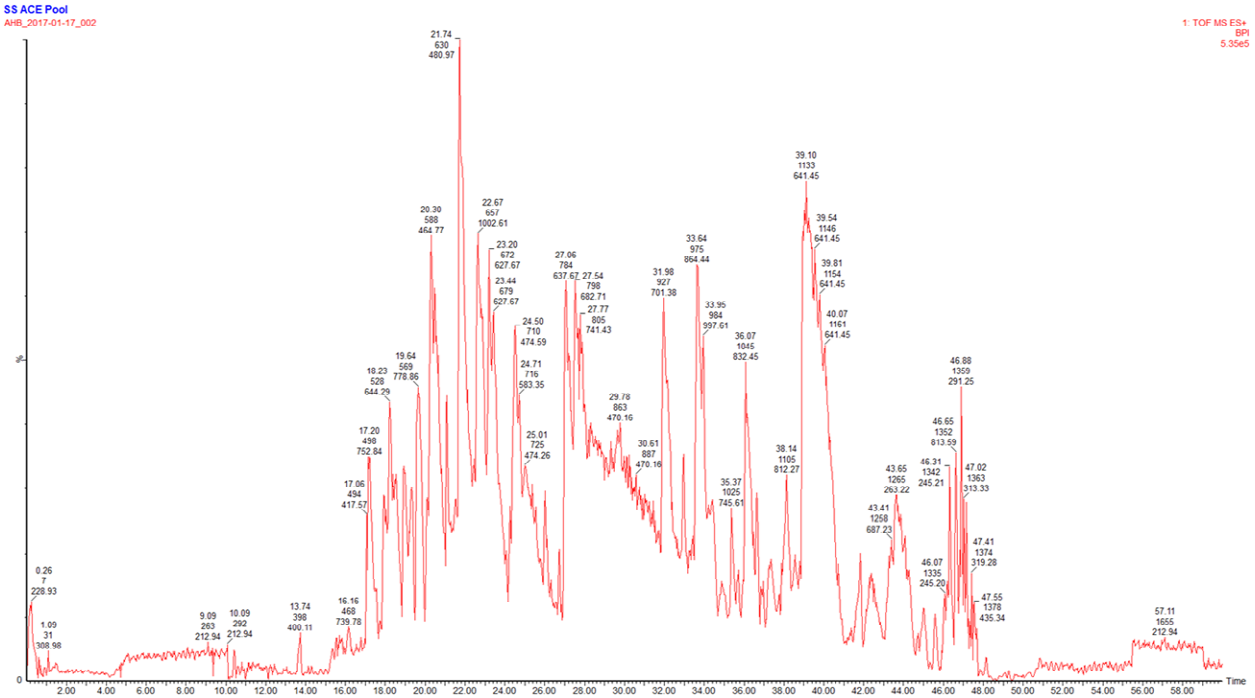

Figure S-10. A representative Baseline Peak Ion Chromatogram of B27-free cell supernatant ultrafiltrated prior digestion.

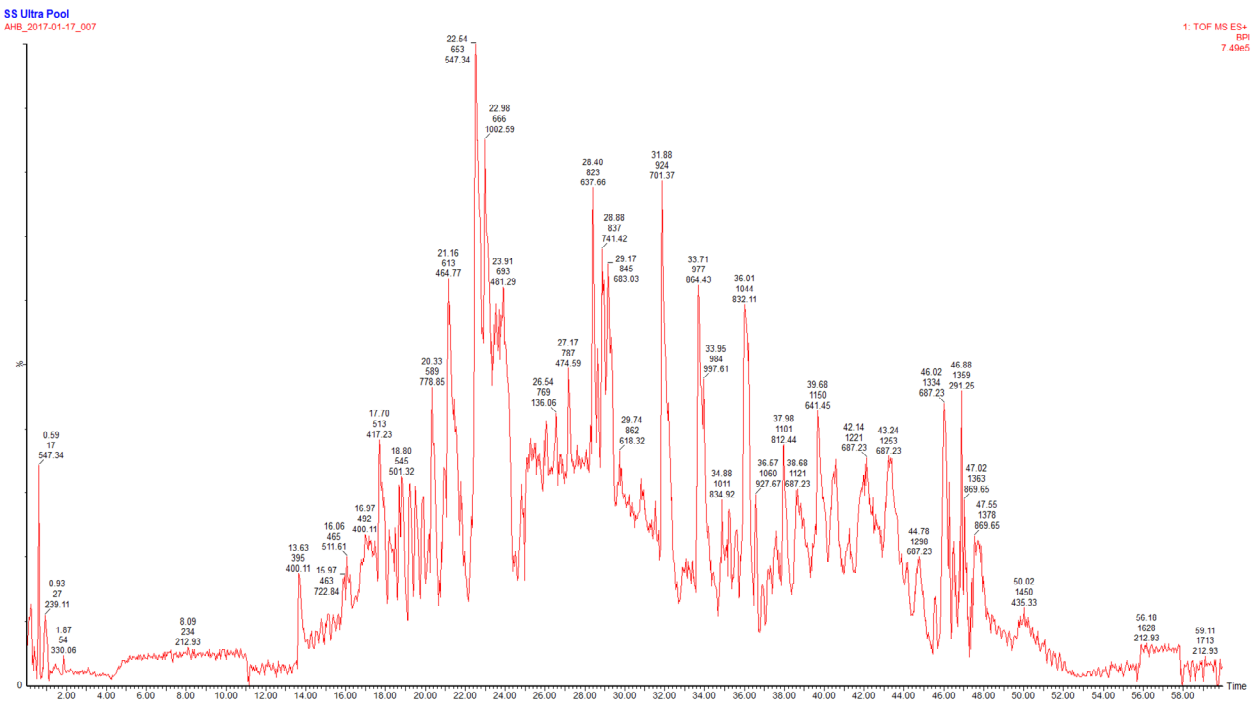

Figure S-11. A representative Baseline Peak Ion Chromatogram of B27-free cell supernatant digested without enrichment step.

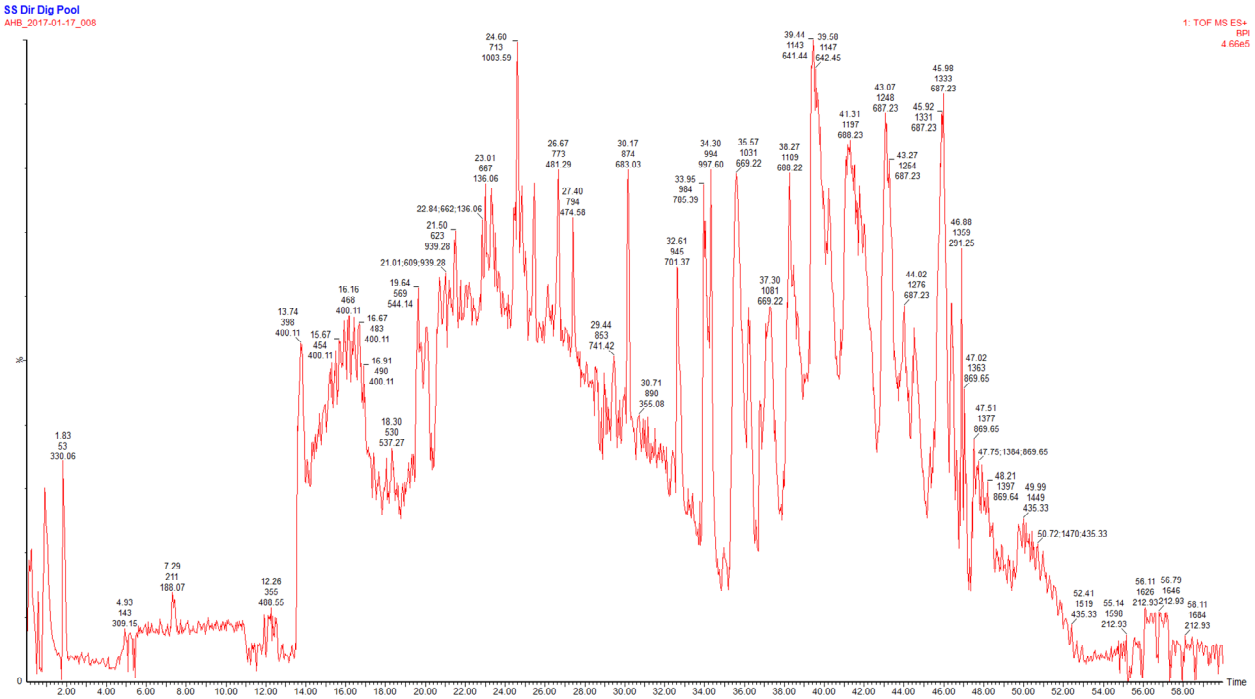

Figure S-12. A representative Baseline Peak Ion Chromatogram of standard cell supernatant precipitated in 10% TCA in acetone prior digestion.

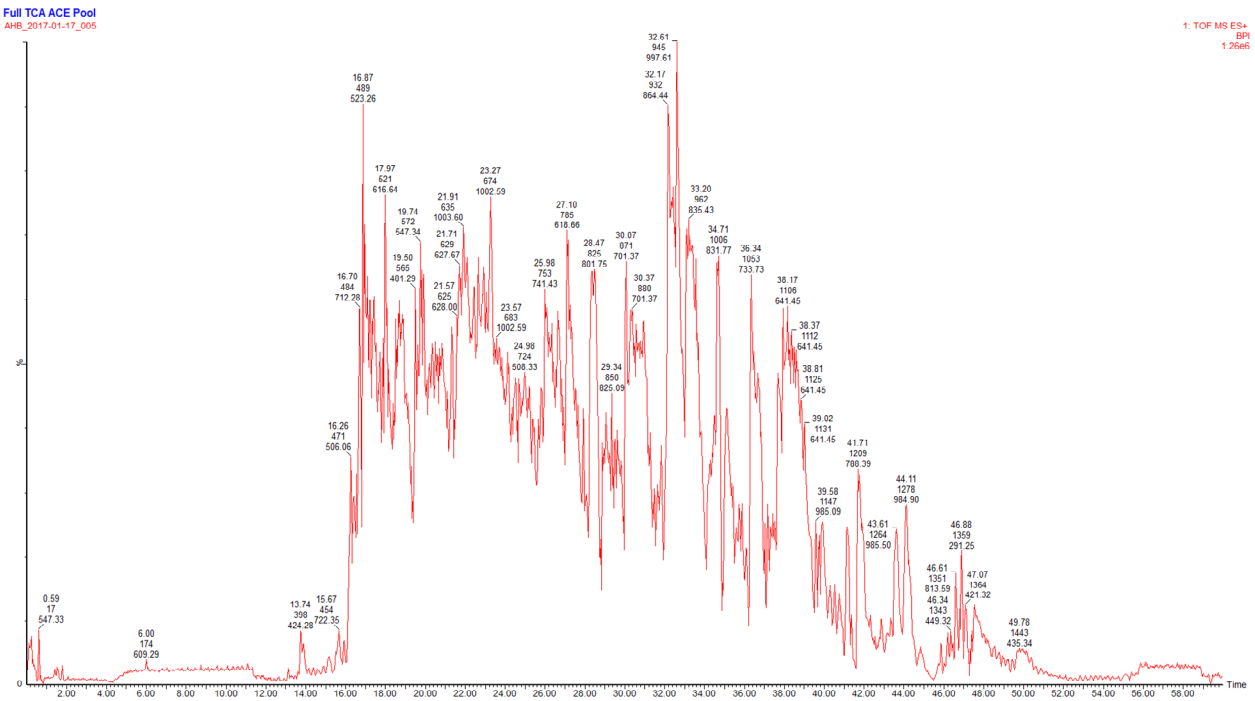

Figure S-13. A representative Baseline Peak Ion Chromatogram of standard cell supernatant precipitated in acetone prior digestion.

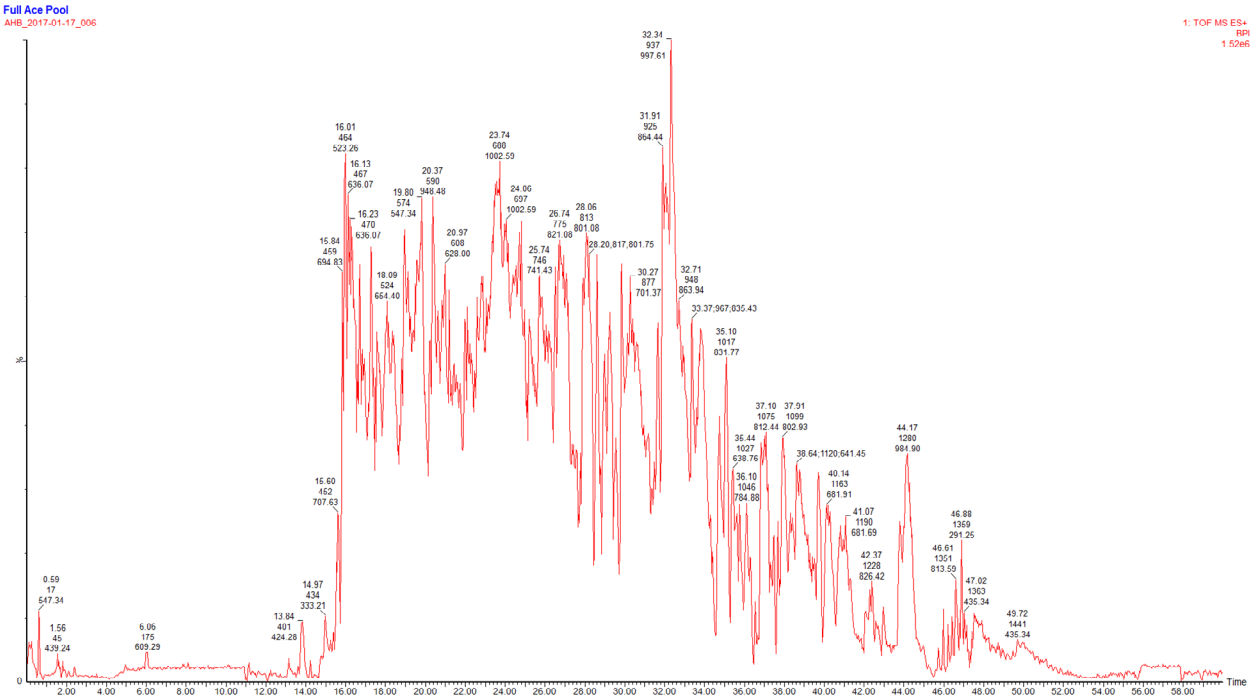

Figure S-14. A representative Baseline Peak Ion Chromatogram of standard cell supernatant ultrafiltrated prior digestion.

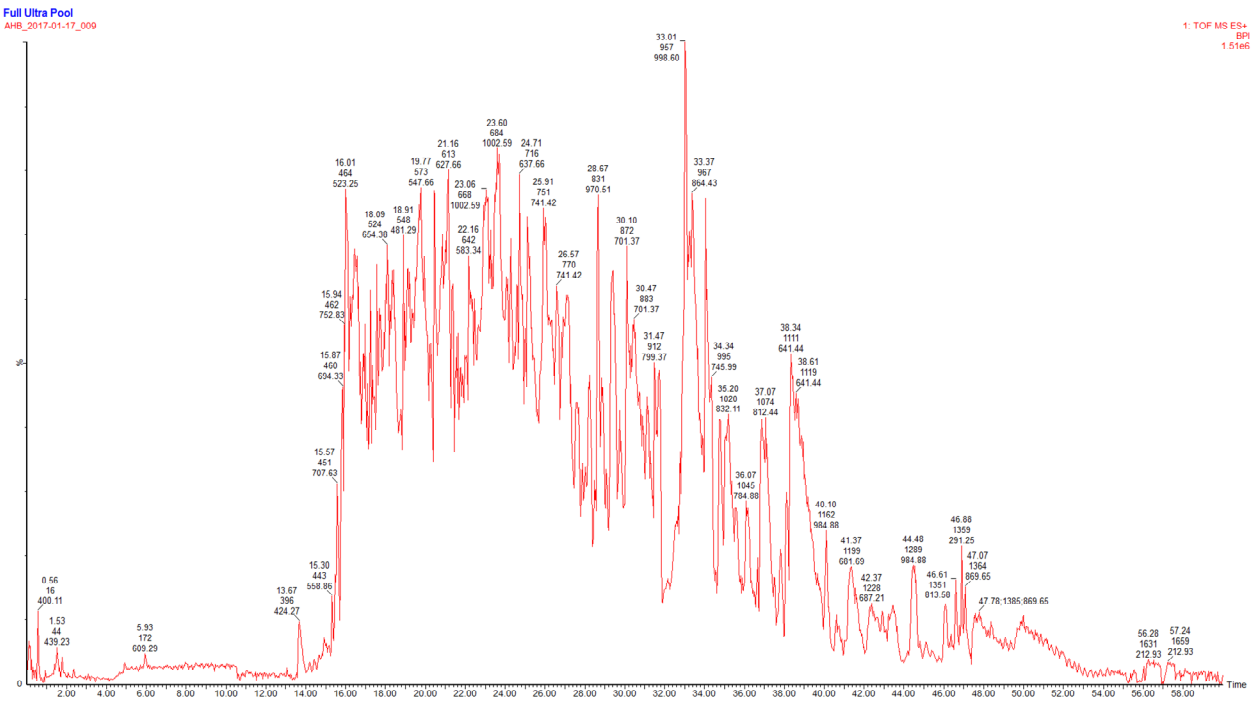

Figure S-15. A representative Baseline Peak Ion Chromatogram of standard cell supernatant digested without enrichment step.

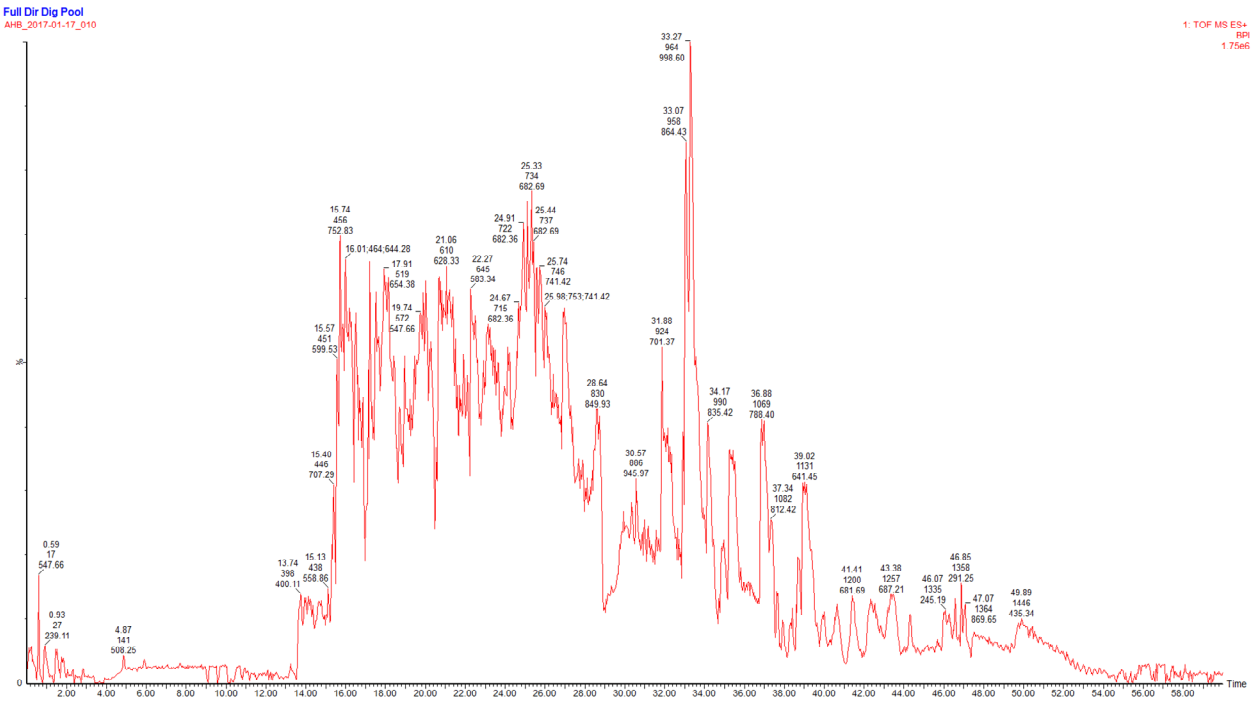

Table S-1. List of cell lines included in the study. A reference is provided for published cell lines.

Cell Line	Sample name	Mutation	Reference
1	SFC840-03-03	Control	Fernandes et al., 2016
2	SBAD04-01	Control	Fernandes et al., 2016
3	SBAD03-01	Control	
4	SBAD03-02	Control	
5	SFC831-03-01	SNCA Triplication	
6	SFC831-03-05	SNCA Triplication	
7	NAS7	Control	Devine et al., 2011
8	AST3	SNCA Triplication	Devine et al., 2011

iPSC lines prefixed with SFC were derived and characterised at the University of Oxford, James Martin Stem Cell Facility. iPSC lines prefixed with SBAD were derived and characterised at the University of Newcastle from Lonza fibroblasts CC-2511, Lot 264781, Tissue Acquisition number 23447. Both were derived as part of the EU IMI-funded programme, StemBANCC, from donors who had given signed informed consent for derivation of hiPSC lines from skin biopsies. NAS and AST iPSC lines were derived from first-degree relatives, with AST carrying the triplication of SNCA.

In the first part of the study, supernatants from iPSC-derived neurons with triplication of SNCA gene locus and unaffected controls (Table S-1) were pooled in order to reduce biological variation. Briefly, 600 μL of each cell supernatant was pooled. Samples of standard and B27-free media were pooled into the separate tubes. Obtained mixtures were vortexed thoroughly and 500 μL was aliquoted to the low protein binding microcentrifuge tubes. Each aliquot was subsequently prepared for proteomic analysis using different method.
In the second part of the study (time-dependent collection), the supernatant of control SFC840-03-03 and patient SFC831-03-05 was used.

Table S-2. List of identified extracellular proteins in cell media conditioned for 3, 6 and 24 h with iPSc-derived neurons.

3h		6h		24h	
Gene name	**Abundance (ng)**	**Gene name**	**Abundance (ng)**	**Gene name**	**Abundance (ng)**
FN1	27.336	TGFBI	60.7713	HSPG2	63.5133
TNC	6.7464	FN1	34.2197	FN1	55.3833
VCAN	2.8073	SPARC	5.63	VCAN	20.589
SPARC	2.4996	TNC	4.5376	TNC	14.7471
TGFBI	0.9097	VCAN	2.781	SPARC	11.8934
SERPINE1	0.5958	MMP2	2.5268	COL5A2	6.411
IGFBP2	0.3081	SERPINE1	0.9234	TGFBI	5.1102
MMP2	0.2283	IGFBP2	0.7789	AGRN	4.5727
		PCSK1N	0.1802	IGFBP2	3.5127
		TIMP1	0.1588	CP	3.2003
		TIMP2	0.1548	MMP2	2.824
				IGFBP5	2.6781
				SERPINE1	2.602
				LUM	1.6879
				SPON1	1.6245
				COL4A1	1.4306
				SERPINH1	1.3586
				SOD1	1.2488
				TIMP1	1.2048
				LAMC1	1.1604
				COL4A2	1.0749
				NTN1	0.9353
				TIMP2	0.9305
				CPE	0.7714
				PSAP	0.6548
				SERPING1	0.5583
				SERPINF1	0.5475
				QSOX1	0.5349

				BMP1	0.5237
				IGFBP4	0.5115
				PCSK1N	0.4957
				MFAP4	0.4413
				VASN	0.4286
				TGFB2	0.3459
				CTGF	0.3403
				CPXM1	0.2936
				COL26A1	0.2072
				CTSB	0.1886

Table S-3. List of identified proteins in Matrigel.

Protein	Gene	Organism	Uniprot code
Laminin subunit gamma-1	Lamc1	Mus musculus	F8VQJ3
Laminin subunit beta-1	Lamb1	Mus musculus	E9QN70
Ceruloplasmin	CP	Homo sapiens	E9PFZ2
Keratin_ type I cytoskeletal 9	KRT9	Homo sapiens	K7EQQ3
Fibrinogen beta chain	FGB	Homo sapiens	D6REL8
Alpha-1-antitrypsin	SERPINA1	Homo sapiens	A0A024R6I7
Collagen alpha-1(IV) chain	Col4a1	Mus musculus	A0A1B0GSI7
Heat shock cognate 71 kDa protein	HSPA8	Homo sapiens	E9PKE3
Complement C3 (Fragment)	C3	Homo sapiens	M0QYC8
Fibrinogen gamma chain	Fgg	Mus musculus	Q3UER8
Uncharacterized protein	LOC400499	Homo sapiens	M0QZD8
Actin_ cytoplasmic 1 (Fragment)	ACTB	Homo sapiens	A0A2R8Y793
Procollagen-lysine_2-oxoglutarate 5-dioxygenase 2	Plod2	Mus musculus	E9Q718
Alpha-1-antichymotrypsin	SERPINA3	Homo sapiens	G3V3A0
Complement C3 (Fragment)	C3	Homo sapiens	M0R0Q9

Antithrombin-II	SERPINC1	Homo sapiens	Q8TCE1
HPX protein	HPX	Homo sapiens	Q9BS19
Complement C3 (Fragment)	C3	Homo sapiens	M0R1Q1
Serpin H1 (Fragment)	Serpinh1	Mus musculus	A0A140LHR4
Uncharacterized protein	Gm20547	Mus musculus	B8JJN0
Fibrinogen gamma chain	FGG	Homo sapiens	C9JC84
Keratin 78	Krt78	Mus musculus	E9Q0F0
Laminin subunit gamma-1 (Fragment)	Lamc1	Mus musculus	F6TLW1
Alpha-1-antichymotrypsin (Fragment)	SERPINA3	Homo sapiens	G3V595
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	Mus musculus	A0A0A0MQF6
Uncharacterized protein	1	Homo sapiens	A0A0B4J269
Transthyretin	TTR	Homo sapiens	A0A087WT59
Transthyretin	TTR	Homo sapiens	A0A087WV45
Basement membrane-specific heparan sulfate proteoglycan core protein	Hspg2	Mus musculus	B1B0C7
ATP synthase subunit beta (Fragment)	ATP5F1B	Homo sapiens	H0YH81
Histone H2B	Hist1h2bq	Mus musculus	Q8CBB6
BSD domain-containing protein 1 (Fragment)	BSDC1	Homo sapiens	E9PQA7
40S ribosomal protein SA	RPSA	Homo sapiens	A0A0C4DG17
Haptoglobin	HP	Homo sapiens	A0A087WU08
Nidogen-2	NID2	Homo sapiens	A0A087WZP6
Vitamin D-binding protein	GC	Homo sapiens	D6RF35
Hexokinase-2	Hk2	Mus musculus	E9Q5B5
Alpha-2-macroglobulin (Fragment)	A2M	Homo sapiens	H0YFH1

Tubulointerstitial nephritis antigen-like	Tinagl1	Mus musculus	H3BJ97
40S ribosomal protein S3	Rps3	Mus musculus	A0A140LI77
Keratin_ type I cytoskeletal 10	Krt10	Mus musculus	A2A513
Insulin	INS	Homo sapiens	A6XGL2
Apolipoprotein A-I	APOA1	Homo sapiens	F8W696
Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	Homo sapiens	F8WAS2
Collagen alpha-1(IV) chain (Fragment)	Col4a1	Mus musculus	A0A1B0GRC0
40S ribosomal protein S14 (Fragment)	RPS14	Homo sapiens	A0A2R8Y811
Elongation factor 1-alpha	EEF1A1	Homo sapiens	A0A087WV01
Tropomyosin alpha-3 chain	TPM3	Homo sapiens	A0A087WWU8
Uncharacterized protein	Rpl7a-ps5	Mus musculus	A0A140T8L3
Beta-globin	Hbb-bs	Mus musculus	A8DUK4
Pyruvate kinase	PKM	Homo sapiens	B4DNK4
Chromosome 12 open reading frame 43	C12orf43	Homo sapiens	F5H7W8
Laminin subunit beta-1	LAMB1	Homo sapiens	G3XAI2
Arf-GAP with SH3 domain_ ANK repeat and PH domain-containing protein 3 (Fragment)	ASAP3	Homo sapiens	H0YE36
40S ribosomal protein S16 OS	RPS16	Homo sapiens	Q6IPX4
MCG23377_ isoform CRA_b	Gm8797	Mus musculus	A0A0A6YW67
Histone H2A	H2afj	Mus musculus	A0A0N4SV66
Periaxin	Prx	Mus musculus	A0A0U1RNK1
Delta(3_5)-Delta(2_4)-dienoyl-CoA isomerase_ mitochondrial (Fragment)	Ech1	Mus musculus	F7B227
Protein FAM107B (Fragment)	Fam107b	Mus musculus	H3BKB6
Serotransferrin (Fragment)	TF	Homo sapiens	H7C5E8

Profilin	Pfn1	Mus musculus	Q5SX49

Table S-4. Differentially expressed proteins by a fold 2 or more between control and patient cell supernatant. All proteins were identified using at least 2 unique peptides.

Protein	Gene	Uniprot code	Fold change	Highest mean observed in the secretome of
Calsyntenin-3	CLSTN3	Q9BQT9	60.25	Control
Clusterin	CLU	P10909	13.60	Control
Transthyretin	TTR	P02766	7.55	Control
Fibronectin	FN1	P02751	7.42	Triplication of SNCA
Heat shock protein HSP 90-alpha	HSP90AA1	P07900	6.48	Control
Prosaposin	PSAP	P07602	5.02	Control
Follistatin-related protein 1	FSTL1	Q12841	4.06	Control
Collagen alpha-1(VI) chain	COL6A1	P12109	3.72	Triplication of SNCA
Alpha-actinin-1	ACTN1	P12814	3.48	Control
Collagen alpha-1(XVIII) chain	COL18A1	P39060	3.38	Control
Collagen alpha-2(I) chain	COL1A2	P08123	3.32	Control
Nucleobindin-1	NUCB1	Q02818	3.08	Control
Thrombospondin-1	THBS1	P07996	3.04	Triplication of SNCA
Collagen alpha-1(II) chain	COL2A1	P02458	3.03	Control
Carboxypeptidase E	CPE	P16870	2.95	Control
Transforming growth factor-beta-induced protein ig-h3	TGFBI	Q15582	2.69	Triplication of SNCA
Keratin, type II cytoskeletal 5	KRT5	P13647	2.55	Triplication of SNCA
Cadherin-2	CDH2	P19022	2.49	Control
SPARC	SPARC	P09486	2.48	Triplication of SNCA
Alpha-actinin-3	ACTN3	Q08043	2.37	Triplication of SNCA
Uncharacterized protein KIAA1109	KIAA1109	Q2LD37	2.34	Triplication of SNCA

Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	P07910	2.26	Triplication of SNCA
Moesin	MSN	P26038	2.25	Triplication of SNCA
Tropomyosin alpha-1 chain	TPM1	P09493	2.21	Triplication of SNCA
L-lactate dehydrogenase B chain	LDHB	P07195	2.11	Control
Serpin H1 OS=Homo sapiens	SERPINH1	P50454	2.06	Triplication of SNCA

Methodology of neuronal differentiation.

iPSCs were differentiated into midbrain dopaminergic neurons as described previously. Briefly, cells were plated at high density on matrigel in iPSC media, then transferred to differentiation media of knockout DMEM with knockout serum replacement (ThermoFisher) supplemented with LDN193189, SB431542, sonic hedgehog C24II, fibroblast growth factor 8a, Purmorphamine + CHIR99021 at various intervals. Cells were passaged using accutase after 21 days, and seeded onto plates coated with poly-L-ornithine, laminin and fibronectin (Sigma). Neurons were matured in Neurobasal media supplemented with B27 and brain-derived neurotrophic factor, glial-derived neurotrophic factor, transforming growth factor beta 3, N2'-O-Dibutyryladenosine 3',5'cyclic monophsphate and ascorbic acid. Cells were passaged approximately 3 more times before being used for experiments at days 50-70.

Methodology of cell viability experiment.

Control iPSC-derived neurons were plated in 96 well plates and maintained in regular media containing B27 for 1 week. The media was then replaced with either B-27 free media or regular media. Following 24 hours incubation CellTitre-Glo reagent was added to wells as to manufacturers protocol and signal was measured on a luminometer. Luminescence of wells containing B27-free media was compared to that of control wells containing regular media.

Preparation of cell supernatants.

Ultrafiltration. 500 μL of sample was placed in Amicon® Ultra-0.5mL centrifugal filter device with 3 kDa cut off (Merck Millipore). Ultrafiltration was performed according to the manufacturer's instructions. After ultrafiltration, the concentrated sample was immediately transferred to the low protein-binding tube and freeze-dried.
Acetone precipitation. 500 μL of sample were mixed with 1500 μL of ice-cold acetone (Sigma) and incubated at -20 ˚C for 15h. Subsequently, samples were centrifuged at 4 ˚C at 13,000 g for 10 min. The resulting supernatant was discarded and the protein pellet was freeze-dried.

MeOH/CH_3Cl precipitation. 500 µL of sample were mixed with 2000 µL of methanol and 500 µL of chloroform and mixed thoroughly. Then, 1500 µL of fresh MilliQ water was added to the solution, vortexed and samples were centrifuged at 13,000 g for 2 min. The upper organic phase was removed without disturbing the interface. 1500 µL of methanol was added to the sample, mixed and centrifuged for 10 min at 5,000 g. Supernatant was discarded and the protein pellet was freeze-dried.

TCA-Acetone precipitation. 500 µL of sample was mixed with 2000 µL of ice-cold 10% Trichloroacetic acid (TCA) in acetone, mixed and incubated at -20 °C for 2h. After the incubation, sample was centrifuged at 4 ˚C at 13,000 g for 10 min. Supernatant was discarded and 1 mL ice-cold acetone was added to the protein pellet. After the wash, sample was centrifuged at 4 ˚C at 13,000 g for 10 min, the supernatant was discarded and the protein pellet was air dried.

Direct Digestion. 500 µL of sample was freeze-dried and submitted to proteomic digestion.

Label-free $UDMS^E$ mass spectrometry. Mass spectrometry analysis was performed as described previously. For each fraction a 60 min mass spectrometry analysis was performed on a SYNAPT G2-Si (Waters, Manchester) mass spectrometer in a UDMSE mode in positive ion electrospray ionisation mode and operated in V-mode. One second alternating high and low energy scans were performed at a capillary voltage of 3.0 kV, sampling cone voltage of 40 V, a source temperature of 70 °C over a mass range of 50–2000 Da in resolution analyser mode. Prior to fragmentation ion mobility separation was performed at a wave velocity of 650 m/s and a wave height of 40 V in order to separate similar precursor ions. The low energy scans were performed at a collision energy of 0 V and the high energy scans were performed on a gradient designed to use an optimised collision energy depending on the ion mobility bin, from 0–20 ion mobility bins the collision energy was 13.6 V increasing linearly to 49.1 V at 120 mobility bins, followed by another linear gradient to 54.1 V at 200 mobility bins. At a frequency of every 60 s lock mass of [glutamic acid1]-fibrinopeptide B was delivered via an auxiliary pump at 300 nL/min.

Quality Control. Each mass spectral analyses was quality controlled (QC) by the addition of QC samples performed after every 10 injections. QC samples included a Peptide Standard Mix (Waters, Manchester) of Ribonuclease pancreatic, Cytochrome c, Serum albumin, Myoglobin, Enolase 1, Glycogen phosphorylase at a concentration of 4.15, 5.65, 20.15, 12.5, 21.45, 58.9 ng/ml, respectively. This peptide QC standard was used to QC chromatographic and mass spectral performance as well as proteomic quantitation. Results of a CV >15% were rejected. In addition, a second QC standard made from pooled samples was used as a biological sample quality control check and was also analysed every 10 samples to control and monitor mass spectral performance and bioinformatics quantitation. Similarly, the monitoring of the 5 main proteins detected in this biological QC followed the same protocol of the standard peptide mix, with a difference in CV of >15% resulted in a batch rejection.

References

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