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Synaptic markers of cognitive decline in neurodegenerative diseases: A proteomic approach.

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ABSTRACT

Cognitive changes occurring throughout the pathogenesis of neurodegenerative diseases are directly linked to synaptic loss. We have used in-depth proteomics to compare 32 post-mortem human brains in the prefrontal cortex of prospectively followed patients with Alzheimer`s disease, Parkinson`s disease with dementia, dementia with Lewy bodies and older adults without dementia. In total, we have identified 10325 proteins, out of which 851 were synaptic proteins. Levels of 25 synaptic proteins were significantly altered in the various dementia groups. Significant loss of SNAP47, GAP43, SYBU, LRFN2, SV2C, SYT2, GRIA3 and GRIA4 were further validated on a larger cohort comprised of 92 brain samples using ELISA or Western blot. Cognitive impairment before death and rate of cognitive decline significantly correlated with loss of SNAP47, SYBU, LRFN2, SV2C and GRIA3 proteins. Besides differentiating Parkinson`s disease dementia, dementia with Lewy body and Alzheimer`s disease from controls with high sensitivity and specificity, synaptic proteins also reliably discriminated Parkinson`s disease dementia from Alzheimer`s disease patients. Our results suggest that these particular synaptic proteins have an important predictive and discriminative molecular fingerprint in neurodegenerative diseases and could be a potential target for early disease intervention.

Keywords: synaptic proteins, cognitive impairment, Lewy body dementias, Alzheimer`s disease, mass spectrometry

Abbreviations: SNAP47-synaptosomal associated protein 47; GAP43-neuromodulin;SYBU-syntabulin; LRFN2-leucine-rich repeat and fibronectin type-III domain-containing protein 2; SV2C-synaptic vesicle 2C; GRIA-glutamate receptor; SYT2-synaptotagmin 2.

1. INTRODUCTION

The pandemic increase of dementia, hampering daily living of many millions, carries serious implications for society (Wimo *et al.*, 2017). Alzheimer's disease and the Lewy body dementias, *i.e.* dementia with Lewy bodies and Parkinson`s disease dementia, are the most common forms of neurodegenerative dementias (Campbell *et al.*, 2001, McKeith *et al.*, 2005).

Cognition gradually declines in Alzheimer`s disease, dementia with Lewy bodies and Parkinson`s disease dementia leading to loss of function in everyday life, reduced quality of life, and increased mortality (Aarsland *et al.*, 2003, McKeith *et al.*, 2005, Maalouf *et al.*, 2011). Synapse and synaptic protein loss seems to be a universal element in the pathologic changes associated with dementia (DeKosky and Scheff, 1990) as it is directly linked to cognitive deficits from early stages of dementia and it is believed that synaptic changes precede neuronal degeneration (DeKosky and Scheff, 1990). It has been shown that synaptic loss is a better correlate of cognitive impairment in Alzheimer`s disease than the hallmark tau and amyloid beta pathologies (Blennow *et al.*, 1996, Masliah *et al.*, 2001). Several studies have shown that changes in synaptic function are associated with alterations in the concentration of synaptic proteins, (Gottschall *et al.*, 2010) a characteristic feature in Alzheimer`s disease (Terry *et al.*, 1991, Honer, 2003) and increasing attention is now being devoted to their role in synucleinopathies (Aarsland *et al.*, 2005, Compta *et al.*, 2011, Howlett *et al.*, 2014, Bereczki *et al.*, 2016). In a recent study we have reported changes in the concentration of presynaptic proteins SNAP25 and Rab3A as well as of postsynaptic protein neurogranin in post-mortem neocortical regions in Parkinson`s disease, dementia with Lewy bodies and Alzheimer`s disease patients. These changes correlated with the rate of cognitive decline in dementia with Lewy bodies and Alzheimer`s disease patients as well as with neuropathological markers (Bereczki *et al.*, 2016). The development of biomarkers aiding early

differential diagnosis and predicting disease progression from its earliest stage is of major importance both for research and therapeutic development. The complex structural and functional organization of the brain regarding its morphology, connectivity and function warrants the application of systematic approaches. Recent advances in mass spectrometry based proteomics offer a reliable molecular phenotype comparison between diseased and control cases allowing in-depth coverage of quantitative changes (Kim *et al.,* 2014). These methods permit the identification of alterations in the cellular proteome and provide insight into disease aetiology and mechanisms. In addition, they aid the discovery of biomarkers for monitoring disease progression as well as assessment of drug effects (Portelius *et al.*, 2015, Moya-Alvarado *et al.*, 2016). Whereas some explorative proteomic studies have already been performed in Alzheimer`s disease and Parkinson`s disease, (Abdi *et al.*, 2006, Blennow and Zetterberg, 2013, Brinkmalm *et al.*, 2014, Halbgebauer *et al.*, 2016) only very few studies have been conducted in dementia with Lewy body (Abdi *et al.*, 2006, Barthelemy *et al.*, 2016, Biemans *et al.*, 2016).

Our study is among the first in-depth quantitative proteome studies on pre-frontal post-mortem tissues where beside the whole proteome comparison we also aimed at profiling the entire synaptic proteome of Alzheimer`s disease, Parkinson`s disease dementia and dementia with Lewy bodies patients and compared them to non-demented control cases. Our in-depth analysis of the synaptic proteome identified key synaptic proteins underlying synaptic dysfunction in Alzheimer`s disease, Parkinson`s disease dementia and dementia with Lewy bodies suggesting shared mechanisms, with major implications for prognostic and diagnostic marker development as well as advancing future therapeutic interventions for improving the disease course.

2. METHODS:

Brain tissue

Post-mortem human brain tissues from prefrontal cortex, Brodmann area 9 (from 92 cases in total) were provided by the Brains for Dementia Research network. The prefrontal cortex was selected due to its role in cognition and executive functions involved across the three diseases(Fuster, 2001). The cohort included cases from the Newcastle Brain Tissue Resource (3 cases), the Thomas Willis Oxford Brain Collections (7 cases), the London Neurodegenerative Diseases Brain Bank (56 cases) and the University Hospital Stavanger (26 cases). Autopsy protocols and sample collection was harmonized between centres. Detailed description of the diagnostic criteria has been previously published (Howlett *et al.*, 2014). Final diagnoses for patients are clinic-pathological consensus diagnoses. In total, 24 Parkinson`s disease dementia patients (age 72–89 years), 26 dementia with Lewy body patients (age 65–91 years), 18 Alzheimer`s disease patients (age 72–103 years) and 24 elderly non-neurological controls (age 65–96 years) were included. Controls did not have significant neurological or psychiatric diseases and presented only mild age-associated neuropathological changes (e.g., neurofibrillary tangle Braak stage II). Semi-quantitative assessments of senile Aβ plaques, phosphotau and α-synuclein pathology were conducted by experienced neuropathologists blind to clinical diagnosis, using a four-tiered scale of 0 (none), 1 (sparse), 2 (moderate) and 3 (severe/frequent) to score sections from each brain area, as described previously (Howlett *et al.*, 2014). Hoehn and Yahr scale was available for 23 out of 24 Parkinson`s disease with dementia patients, and assessment from the last off phase was utilized. Alzheimer`s disease patients with low α-synuclein pathology were chosen to ensure distinction between Alzheimer`s disease and dementia with Lewy body patients. Lewy body dementia cases selected were of pathologically 'diffuse neocortical' stage, with a cortical Lewy body score of 13.2 (±3.6), incorporating the 1-year rule to differentiate between dementia with Lewy bodies and Parkinson`s

disease with dementia (McKeith *et al.*, 2005). Neuropathologic assessment was performed according to standardized neuropathologic scoring/grading systems; assessment and diagnostic criteria have been previously described (Howlett *et al.*, 2014). Cognitive data was available for most patients and consisted of the last Mini-Mental State Examination (MMSE) scores, assessed in most cases within 1-2 years before death (Folstein *et al.*, 1975) and MMSE decline calculated as average decline over a period of clinical observation of 8–10 years. All participants gave informed consent for their tissue to be used in research and the study was approved by the UK National Research Ethics Service (08/H1010/4), the Norwegian committee for medical and health research ethics (2010/633) and the Regional Ethical Review Board of Stockholm (2012/920-31/4).

Sample preparation for HiRIEF LC-MS proteomics

The tissues were lysed in SDS-lysis buffer (4% (w/v) SDS, 25mM HEPES pH 7.6, 1mM DTT). Lysates were then heated at 95ºC for 5min in a thermomixer, and were sonicated with a sonicator probe to shear DNA. Samples were centrifuged at 14000 *g* to remove cell debris, the supernatant was collected and protein concentration estimated by the DC-protein assay (BioRad). From each sample, 250µg of total protein were taken and processed according to the FASP (Filter Aided Sample Preparation) protocol (Wisniewski *et al.*, 2009) with one modification, *i.e.* the samples were digested on the filter with Lys-C for 3 hours prior to trypsin digestion (16h). Peptide concentration was estimated by the DC-protein assay (BioRad), and 100µg of peptides from each sample were labelled with the respective TMT10plex reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

HiRIEF (High resolution isoelectric focusing) separation

Peptide pre-fractionation was done using HiRIEF (Branca *et al.*, 2014). Briefly, after pooling the samples that belong together in each TMT set, each TMT set was cleaned by strong cation exchange solid phase extraction (SCX-SPE, Phenomenex Strata-X-C, P/N 8B-S029-TAK). After drying in a SpeedVac (Thermo SPD111V with refrigerated vapor trap RVT400), the equivalent to 400µg of peptides of each sample were dissolved in 250µl of 8M urea, 1% pharmalyte (broad range pH 3-10, GE Healthcare, P/N 17-0456-01), and this solution was used to rehydrate the IPG drystrip (pH 3-10, 24 cm, GE Healthcare, P/N 17-6002-44) overnight. Focusing was done on an Ettan IPGphor 3 system (GE Healthcare), ramping up the voltage to 500V in one hour, then to 2000V in two more hours, and finally to 8000V in six more hours, after which voltage was held at 8000V for additional 20h or until 150kVh were reached. After focusing was complete, a well-former with 72 wells was applied onto each strip, and liquid-handling robotics (GE Healthcare prototype modified from a Gilson liquid handler 215), using three rounds of different solvents (i. milliQ water, ii. 35% acetonitrile, and iii. 35% acetonitrile, 0.1% formic acid), added 50µL of solvent to each well, waited 30 min incubating, and finally transferred the 72 fractions into a microtiter plate (96 wells, polypropylene, V-bottom, Greiner P/N 651201), which was then dried in a SpeedVac.

LC-MS analysis

For each LC-MS run of a HiRIEF fraction, the auto sampler (Ultimate 3000 RSLC nanoUPLC system, Thermo Scientific Dionex) dispensed 15µl of mobile phase A (95% water, 5% dimethylsulfoxide (DMSO), 0.1% formic acid) into the corresponding well of the microtiter plate, mixed by aspirating/dispensing 10µl ten times, and finally injected 7µl into a C18 guard desalting column (Acclaim pepmap 100, 75µm x 2cm, nanoViper, Thermo). After 5min of flow at 5µl/min

with the loading pump, the 10-port valve switched to analysis mode in which the NC pump provided a flow of 250nL/min through the guard column. The curved gradient (curve 6 in the Chromeleon software) then proceeded from 3% mobile phase B (90% acetonitrile, 5% DMSO, 5% water, 0.1% formic acid) to 45% B in 50min followed by wash at 99%B and re-equilibration. Total LC-MS run time was 74min. We used a nano EASY-Spray column (pepmap RSLC, C18, 2µm bead size, 100Å, 75µm internal diameter, 50cm long, Thermo) on the nano electrospray ionization (NSI) EASY-Spray source (Thermo) at 60ºC. Online LC-MS was performed using a hybrid Q-Exactive mass spectrometer (Thermo Scientific). FTMS master scans with 70,000 resolution (and mass range 300-1600 m/z) were followed by data-dependent MS/MS (35,000 resolution) on the top 5 ions using higher energy collision dissociation (HCD) at 30% normalized collision energy. Precursors were isolated with a 2m/z window. Automatic gain control (AGC) targets were 1e6 for MS1 and 1e5 for MS2. Maximum injection times were 100ms for MS1 and 150ms for MS2. The entire duty cycle lasted ~1.5s. Dynamic exclusion was used with 60s duration. Precursors with unassigned charge state or charge state 1 were excluded. An underfill ratio of 1% was used.

Proteomics database search

All MS/MS spectra were searched by MSGF+/Percolator using a target-decoy strategy. Raw MS/MS files were converted to mzML format using msconvert from the ProteoWizard tool suite(Kessner *et al.*, 2008). Spectra were then searched using MSGF+ (Kim and Pevzner, 2014) (v10072) and Percolator (Kall *et al.*, 2007) (v2.08), where 8 subsequent search results were grouped for Percolator target/decoy analysis. The reference database that was used was the human subset of the Swiss-Prot database (version 2015_08, with 42122 canonical and isoform protein entries, downloaded from uniprot.org). MSGF+ settings included precursor mass tolerance of

10ppm, fully-tryptic peptides, maximum peptide length of 50 amino acids and a maximum charge of 6. Fixed modifications were TMT-10plex on lysine residues and N-termini, and carbamidomethylation on cysteine residues; a variable modification was used for oxidation on methionine residues. Peptide and PSM FDR were recalculated after merging the percolator groups of 8 search results into one result per TMT set. Quantification of TMT-10plex reporter ions was done using OpenMS project's IsobaricAnalyzer (Rost *et al.*, 2016) (v2.0). PSMs found at 1% PSM- and peptide-level FDR (false discovery rate) were used to infer gene identities, whose respective protein products were quantified using the medians of PSM quantification ratios, which were subsequently normalized to the median protein value of each TMT channel ratio. Only one unique peptide was required to identify a protein, but a protein level FDR cut-off of 1% (calculated using the picked-FDR method (Savitski *et al.*, 2015)) was applied to the list of gene-centered proteins. Thus, all PSMs, peptides and proteins included in the final results were filtered through both a 1% peptide level FDR and a 1% protein level FDR. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD006122.

Gene ontology and pathway enrichment analyses

Gene ontology (GO) terms were retrieved from uniprot.org for all proteins identified. Proteins with GO terms (in all ontologies: biological processes, molecular function, cellular component) containing the word “synapse” or “synaptic” were considered synaptic proteins and used for further enrichment analysis. T-tests comparing the sample groups using log2-transformed ratios were used to determine whether proteins were differentially accumulated (requirements: $p<0.05$ and fold change <0.83 or >1.20, which is based on the 95% confidence interval of the variance

between the two replicate internal pooled standard TMT channels). The proteins deemed significant were then assigned to Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis using DAVID Bioinformatics Resources version 6.8 (Huang da *et al.*, 2009). An EASE score (a modified Fisher's exact test) <0.1 and p-value <0.05 were the criteria for significantly enriched biological pathways. The GOrilla (Gene ontology enrichment analysis and visualization) tool(Eden *et al.*, 2009) was used for detailed data analyses with two unranked lists of genes, a target list and a background list (composed by all genes identified at protein level in the MS experiment), with a GO database from 2017-01-21. Results were categorized into the functional groups of cellular component, biological process or molecular function. FDR q-values of <0.5, representing the correction of p-values for multiple testing, were considered significant.

Preparation of tissue samples for Western blotting and ELISA

Preparation of tissue for western blotting and ELISA analyses was performed as previously described (Kirvell *et al.*, 2006). Briefly, 500 mg of frozen tissue was homogenized in ice-cold buffer containing 50 mM Tris-HCL, 5 mM EGTA, 10 mM EDTA, protease inhibitor cocktail tablets (Roche, 1 tablet per 50 mL of buffer), and 2 mg/mL pepstatin A dissolved in ethanol:dimethyl sulfoxide 2:1 (Sigma). The buffer was used at a ratio of 2 mL to every 100 mg of tissue, and homogenization was performed using an IKA Ultra-Turrax mechanical probe (IKA Werke, Germany) until the liquid appeared homogenous. Protein concentration of each sample was measured by using BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific). Samples for ELISA measurements were further diluted to 0.5 μg/ μL total protein in PBS buffer (phosphate buffer saline). Parts of Figure 1 and Figure 4 were crafted in the Mind the Graph platform.

Sandwich enzyme-linked immunosorbent assays

Commercial sandwich ELISA kits for each of the selected synaptic proteins were purchased from Mybiosource. Assay procedures were followed according to the manufacturer`s protocol. 50 µl of standard samples for SV2C and SNAP47 and 100 µl in case of GRIA3, GRIA4 and GAP43 were incubated with the corresponding HRP-conjugate reagent for 1h at 37ºC, followed by thorough washing steps. Chromogen solution was then applied and after stopping the reaction, absorbance measured immediately at 450 nm on a SpektraMax Plus384 microplate reader (Molecular Devices). The sigmoidal standard was evaluated with non-linear four-parameter fit using SoftMax Pro 5.2 software and sample amounts were obtained using the fitted standard curve. Standards and samples were measured in duplicates. Samples of human brain were added in dilutions of 0.5 µg/µL of total protein and standards were diluted so that the sample absorbance values would fall near 50% binding (the linear range) of the standard curve. Concentrations were calculated after the mean blank value had been subtracted.

Immunoblotting

To minimize inter-blot variability, 20 µg of total protein per sample was loaded in each lane on 7.5-10% SDS-polyacrylamide gels (Criterion) for protein separation and then transferred to nitrocellulose membrane (Immobilon-P, Millipore). Each gel contained a control lane of pooled brain homogenates used as an internal standard. After blocking non-specific binding, membranes were incubated with primary antibodies (**Supplementary Table 1**) followed by HRP conjugated secondary antibody. GAPDH was used as a reference protein assessing equal loading. Bands were visualized using Chemiluminescent substrate (Millipore) in a LAS-3000 luminescent image reader

(Fujifilm). Western blot data were evaluated and quantified using Multi Gauge Image Analyzer (version 3.0).

Statistical analysis

To compare synaptic protein levels between groups, Student's T-tests were applied on log2-transformed data using SAM (Significance Analysis of Microarrays) under R (version 3.2.2, The R Foundation for Statistical Computing). SAM performs t-tests using permutation based corrections for multiple comparisons. Although originally designed for array data, SAM has been shown to be valid also for LC-MS/MS data (Roxas and Li, 2008, Sandberg *et al.*, 2012). Additional univariate analyses were carried out using nonparametric statistical tests due to the irregular non-Gaussian distribution of the samples in SPSS (IBM Statistics 22). To assess the relationship between synaptic proteins, and neuropathological and MMSE scores, Spearman correlations were performed. To compare protein levels between controls and the different patient groups we used Kruskal-Wallis tests, followed by Dunn's post hoc test. In all cases, differences were considered statistically significant at $p \leq 0.05$. Chi-squared tests (with Yates Continuity Correction) were used to explore differences in gender across diagnostic groups. A linear regression for the correlation studies was applied to synaptic proteins in order to regress out the effects of age. Prior to linear regression, logarithmic normalization was applied to synaptic proteins to achieve normality.

Multivariate data analyses were performed in order to discriminate controls from the different patient groups using orthogonal partial least square analyses (OPLS in the program SIMCA, version 13.0; Umetrics AB, Sweden). Detailed description of the multivariate statistical analysis can be found in the supplementary methods section. We calculated the sensitivity, specificity,

positive predictive values and negative predictive values of the group separations from the $Q^2(Y)$ values obtained in each model.

3. RESULTS

Demographic characteristics of the samples

Key cohort characteristics are shown in Table 1. In the mass spectrometry studies we have included a total of 32 (8 non demented/ 8 Parkinson`s disease dementia/ 7 dementia with Lewy bodies/ 9 Alzheimer`s disease) patients, while in the validation studies a total of 92 (24 non-demented/ 24 Parkinson`s disease dementia/ 26 dementia with Lewy bodies/ 18 Alzheimer`s disease) patients were included. There were no significant differences in the pH (Chi Square=6.147, df=3, p=0.105) or in the post-mortem delay between the groups (Chi Square=5.037, df= 3, p=0.169). In the larger cohort, Alzheimer`s disease patients were significantly older than all the other three groups (p =0.001 versus controls; p=0.019 versus Parkinson`s disease dementia; p=0.005 versus dementia with Lewy bodies) while Parkinson`s disease dementia and dementia with Lewy bodies patients did not differ significantly in age. dementia with Lewy body and Alzheimer`s disease had longer dementia duration than Parkinson`s disease dementia (p=0.006 Parkinson`s disease dementia versus dementia with Lewy body; p=<0.001 Parkinson`s disease dementia versus Alzheimer`s disease; p=0.002 versus Alzheimer`s disease). Correlations between age and MMSE decline scores were observed in Parkinson`s disease dementia (Rho=0.553, p=0.008, n=22). The last MMSE scores before death were lower and the rate of MMSE decline was higher in Alzheimer`s disease compared to Parkinson`s disease dementia or dementia with Lewy body. No significant association was observed between diagnosis and gender.

Proteome analyses

In order to avoid large inter-individual variation, a confounding factor in previous comparative proteomic studies on clinical material, we included a large number of cases per disease type (7-9). The 32 prefrontal cortex samples were processed by Filter Aided Sample Prep (FASP)(Wisniewski *et al.*, 2009) and cases were individually labelled at peptide level with four sets of isobaric tags (TMT10plex). Each TMT set contained eight channels with randomized samples (**Supplementary Table 2**) and two channels with the internal reference sample (made by pooling aliquots from all 32 samples). Each TMT set was fractionated into 72 fractions by High Resolution IsoElectric Focusing (HiRIEF)(Branca *et al.*, 2014) with the broad range IPG 3-10 strip prior to LC-MS analysis (**Fig. 1a**).

A total of 10325 proteins (gene-centric) were identified (1% False Discovery Rate, protein level FDR) (of which 7033 were common to all 32 samples) as a result of the proteomic database search (**Fig. 1a**). The DAVID (The Database for Annotation, Visualization and Integrated Discovery) platform as well as the GOrilla (Gene ontology enrichment analysis and visualization) tool were used for detailed data analyses. Across the disease groups in total 102 proteins were commonly differentially regulated (**Fig. 1b**). In the dementia with Lewy body group, 1010 differentially expressed proteins (out of which 448 were up-regulated and 562 were down-regulated in dementia with Lewy body compared to non- demented controls) were introduced in the DAVID platform. Of these, 392 were assigned to 22 predicted KEGG pathways (Kyoto Encyclopaedia of Genes and Genomes), with the identified differentially accumulated proteins found to be enriched in pathways related to human diseases (40%), organismal systems (30.3%), cellular processes (10.6%), genetic

information processing (9.2%), metabolism (8.3%) and environmental information processing (1.9%) (**Fig. 1c** and **Supplementary Table 3**). Interestingly, pathways such as Parkinson`s disease (N=22), Alzheimer`s disease (N=24) and Huntington disease (N=27), dopaminergic synapses (N=17), protein processing in endoplasmic reticulum (N=27) and oxidative phosphorylation (N=23) were significantly enriched with the highest number of alterations (**Fig. 1c** and **Supplementary Table 3**). In Parkinson`s disease dementia, a total of 485 proteins (286 up-regulated and 199 down-regulated compared to non-demented controls) were introduced in the DAVID platform, out of which 182 were assigned to 8 KEGG pathways. In Alzheimer`s disease, out of the 593 (255 up-regulated and 338 down-regulated compared to non-demented controls) proteins introduced, 241 were assigned to 12 KEGG pathways. Since there were generally fewer than 10 hits for the pathways, neither Parkinson`s disease dementia nor Alzheimer`s disease KEGG pathway enrichment was further scrutinized (data not shown). For the gene ontology (GO) analyses, in the case of dementia with Lewy body, 1003 proteins with GO terms were assigned to the annotated 1010 proteins using the GOrilla tool and classified into three groups (biological process, molecular function and cellular component) (**Fig. 1d**). Within the three main categories, only significant classifications were found in the cellular compartment (FDR, $q<0.05$), and these were related to organelles and mitochondria (**Fig. 1d** and **Supplementary Table 4**). In Alzheimer`s disease, the only significant hits were found for the molecular function category related to translation initiation and RNA binding, while in Parkinson`s disease dementia no significant classification was found (**Supplementary Table 4**).

Synaptic dysfunction in Parkinson`s disease dementia, dementia with Lewy body and Alzheimer`s disease

Using gene ontology terminology we identified a total of 851 proteins related to synaptic transmission (**Supplementary Table 5**), out of which 25 synaptic proteins were significantly altered in the various dementias as shown by the SAM (Significance Analysis of Microarrays) analyses with low FDR (q<3.5 %). As the levels of CAMK2 and neurogranin have already been assessed in this cohort(Vallortigara *et al.*, 2014, Bereczki *et al.*, 2016), we selected eight additional differentially regulated synaptic proteins based on their function, fold change, and antibody availability, for further validation on a larger cohort (containing the mass spectrometry cohort) using ELISA or Western blot analyses. The synaptic protein with the most conspicuous drop in concentration (29%-33%) was LRFN2 in all three dementias (**Table 3** and **Fig. 2a**). In Parkinson`s disease dementia, SNAP47 and SYT2 concentrations (**Supplementary Fig. 1**) also decreased compared to controls while GAP43 concentration decreased in comparison to the Alzheimer`s disease group. Five out of the eight measured synaptic proteins were decreased in dementia with Lewy body compared to non-demented controls. In addition to concentrations of LRFN2, SNAP47 and SV2C, levels of SYBU and SYT2 were also decreased (**Table 3, Fig. 2a** and **Supplementary Fig. 1).** In Alzheimer`s disease, apart from LRFN2, only GRIA3 concentration was significantly decreased (**Table 3** and **Fig. 2a**). Proteomic profiling revealed no significant differences in α-synuclein levels (**Supplementary Table 5**).

Panel of synaptic proteins discriminate between control and dementia diagnoses

Multivariate analyses showed that synaptic protein levels were able to provide a clear separation between controls and the different patient groups however no synaptic protein alone was able to achieve clear discrimination between groups. In addition, a clear discrimination between Parkinson`s disease dementia and Alzheimer`s disease patients was also observed (77.8%

sensitivity with 80% specificity, **Table 4**). All models were statistically significant and showed sensitivity, specificity, positive and negative predictive values that were above 80% in the case of dementia with Lewy body versus control and Alzheimer`s disease versus control groups, and were close to 75% in Parkinson`s disease dementia patients versus controls (**Table 4**).

The model comparing control with Parkinson`s disease dementia cases showed a modest predictive power of Q2(Y) = 0.173 in discriminating controls from patients with Parkinson`s disease dementia. With the exception of GRIA4 and SYBU, all variables contributed to the separation between these groups (**Fig. 2b**). The dementia with Lewy body model showed a good predictive power of Q2(Y) = 0.471 in discriminating controls from dementia with Lewy body patients. All synaptic proteins significantly contributed to the separation between groups, with the exception of GRIA4 and GAP43 (**Fig. 2c**). The Alzheimer`s disease model showed a good predictive power of Q2(Y) = 0.427 in the discrimination of controls from Alzheimer`s disease patients. The synaptic proteins that significantly contributed to the separation were LRFN2, GRIA3, SV2C and SYT2 (**Fig. 2d**). A good predictive power of Q2(Y) = 0.438 reflected the capacity to distinguish Parkinson`s disease dementia and Alzheimer`s disease pathology based on the contribution of both GAP43 and SNAP47 (**Fig. 2e**). These results were still significant after correcting for the effects of age. Parkinson`s disease dementia and dementia with Lewy body patients could not be reliably discriminated from one another, supporting that they are part of the same disease spectrum. No significant differences were found between dementia with Lewy body and Alzheimer`s disease most likely attributed to the common amyloid related pathology.

Associations between synaptic proteins and neuropathological scores

Correlations between the eight synaptic proteins validated on the larger cohort and Alzheimer`s disease and dementia with Lewy body regional pathologies were analysed. (**Supplementary Table 6)**. In Parkinson`s disease dementia there were significant correlations between α-synuclein and SNAP47 (Rho=-0.539, p=0.008) and GRIA3 (Rho=-0.449, p=0.047) whereas in dementia with Lewy body α-synuclein correlated with SV2C (Rho=-0.441, p=0.035). Amyloid β scores correlated significantly with GRIA4 both in Parkinson`s disease dementia (Rho=-0.471, p=0.031) and in dementia with Lewy body (Rho=-0.444, p=0.05). The only significant association we found between synaptic proteins and tangle scores was in the case of GRIA3 in Parkinson`s disease dementia (Rho=-0.460, p=0.041). No neuropathological associations were found in Alzheimer`s disease.

Correlations between synaptic proteins and cognitive impairment

We explored whether synaptic protein changes were associated with cognitive impairment. Due to the exploratory nature of these analyses and small number of patients per group, these correlations are presented without adjusting for multiple comparisons. Our calculations revealed that only results at $p < 0.0087$ would be considered statistically significant with FDR corrections, which is quite a stringent threshold. Significant associations between synaptic proteins and cognitive decline were found in Parkinson`s disease dementia, dementia with Lewy body and in Alzheimer`s disease (**Fig. 3** and **Supplementary Table 7**). Synaptic vesicle protein SV2C was strongly associated with the rate of cognitive decline, i.e. reduced levels correlated with faster cognitive decline in Parkinson`s disease dementia (Rho=-0.486, p=0.022) and dementia with Lewy body (Rho=-0.889, p=0.0001) and low last MMSE score in dementia with Lewy body (Rho=0.759, p=0.0001) (**Fig. 3a,b**). Decrease in SNAP47 concentration was associated with worsening

cognition reflected both by last MMSE scores (Rho=0.480, p=0.027) and the rate of MMSE decline (Rho=-0.559, p=0.008) in Parkinson`s disease dementia. GRIA 3 also presented associations both with cognitive decline (Rho=-0.726, p=0.0001) and last MMSE scores (Rho=0.479, p=0.033) in dementia with Lewy body. Furthermore, in dementia with Lewy body lowered SYBU levels correlated with lower MMSE scores (Rho=0.493, p=0.023). In Alzheimer`s disease only LRFN2 presented strong associations with worsening cognition (Rho=-0.613, p=0.012) and the last MMSE scores (Rho=0.730, p=0.001) (**Fig. 3c**). No significant associations were found between MMSE scores and GRIA4, SYT2 or GAP43 proteins, however GAP43 was found to be associated to the total years of dementia in Alzheimer`s disease (Rho 0.499, p=0.035). No associations were found between motor symptoms and synaptic proteins in Parkinson`s disease with dementia patients (data not shown). The results remained significant after controlling for the effects of age (data not shown).

4. DISCUSSION

Both Alzheimer`s disease and Lewy body diseases are characterised by substantial synaptic loss which so far serves as the best correlate with cognitive impairment (Terry *et al.*, 1991, Scheff *et al.*, 2007, Pienaar *et al.*, 2012). More than 1,000 proteins participate in the finely tuned process of synaptic transmission, a process which comprises interactions between synaptic vesicle membrane proteins as well as pre-synaptic and post- synaptic membrane proteins (Sudhof and Rothman, 2009). While general synaptic loss is a common feature of dementia, specific pre and postsynaptic proteins such as Rab3A, SNAP25, synaptophysin crucial for vesicle trafficking, exo- and endocytosis have been found specifically altered in neurodegenerative diseases (Whitfield *et al.*, 2014, Bereczki *et al.*, 2016) along with NMDA and AMPA receptors, PSD95 and neurogranin

(Whitfield *et al.*, 2014, Bereczki *et al.*, 2016) (Lipton and Rosenberg, 1994, Lee *et al.*, 2008) playing a role in long-term potentiation (**Fig. 4**). Of note, in our current proteomics study we have not identified significant changes neither for SNAP25 nor for Rab3a, which could be due to the relative vulnerability of Rab3a to post mortem delay time (Ferrer *et al.*, 2007). The potential use of key synaptic proteins as biomarkers has recently become in the spotlight of discussions in various dementias (Bereczki *et al.*, 2016, Wellington *et al.*, 2016). Worth mentioning that regional specific post-mortem synaptic protein profile might differ from the synaptic protein profile of cerebrospinal fluid (Bereczki *et al.*, 2016, Remnestal *et al.*, 2016, Bereczki *et al.*, 2017). Although cerebrospinal fluid biomarkers are most informative in portraying the biochemical picture of the brain, blood-based biomarkers are more desired for large-scale screening (Mattsson *et al.*, 2015). Of note, even if a biomarker has shown high specificity and sensitivity, its utility as a theragnostic biomarker is not guaranteed (Mattsson *et al.*, 2015).

The technological advance in proteomics analyses has provided high-throughput screening methods in the quest for biomarkers of neurodegenerative disorders in post-mortem as well as in cerebrospinal fluid or blood based samples. To our knowledge, we are the first to provide a systematic proteome profile comparison on post-mortem human brains (N=8 C, N=8 Parkinson`s disease dementia, N=7 dementia with Lewy body, N=9 Alzheimer`s disease, *i.e.* 32 samples in total) from the prefrontal cortex (Brodmann area 9), revealing a pattern of synaptic protein loss across different neurodegenerative diseases.

We have adopted a proteomics-driven discovery approach and after identifying roughly half of the human proteome, we validated lead synaptic candidates in a larger post-mortem brain cohort of 92 cases. Comparative proteomics highlighted significant loss of several synaptic proteins across dementias including presynaptic proteins (GRIK2, CAMK2A, BDNF, PDYN), synaptic vesicle

priming proteins (SNAP47) synaptic vesicle proteins (SV2C, SYT2), proteins found in both pre and postsynaptic terminus (GAP43, LRFN2) and postsynaptic proteins (GRIA3, GRIA4, ARC, CNIH2, PVRL3, NRGN). Among these proteins, SNAP47, SV2C, GRIA3, SYBU and LRFN2 in the prefrontal cortex correlated with cognitive decline in demented cases. The levels of apocalmodulin-binding proteins, NRGN and GAP43 diminished, which in turn might further contribute to the altered CAMK2 and AMPA receptor (GRIA3, GRIA4) mediated synaptic transmission. Their reduction reflects a selective alteration in a subset of synaptic proteins, suggesting that a decline in synaptic function rather than synaptic loss plays a more relevant role in contributing to dementia progression.

However, the mechanisms leading to synapse destabilization and neuronal death remain elusive. There is evidence showing that synaptic plasticity underlying learning and memory often involves activity-dependent recruitment of synaptic AMPA receptors (GRIA) (Nicoll and Malenka, 1999, Kandel, 2001). During long-term potentiation, GRIA exocytosis is mediated by Q-SNARE proteins syntaxin-3 and SNAP-47 (Jurado *et al.*, 2013). Dysregulation of AMPA receptors has also been implicated in numerous neurodegenerative and psychiatric disorders (Lipton and Rosenberg, 1994). Likewise, deletion of LRFN2 (Leucine-rich repeat and fibronectin III domain-containing 2) localized both to the presynaptic and postsynaptic membrane has been linked to selective working memory and executive deficits, impaired intellectual functioning and auditory-verbal problems (Thevenon *et al.*, 2016). Additional proteins with a potential role in cognitive impairment such as the members of the SNARE family, synaptotagmins (SYT), PSD95 and synaptic vesicle 2 (SV2) proteins have also been incriminated in this captivatingly complicated process of synaptic plasticity (Bajjalieh *et al.*, 1994, Xu *et al.*, 2007, Dun *et al.*, 2010, Whitfield *et al.*, 2014) (**Fig. 4**).

In line with this, SYT2 levels have also been shown to be decreased in plasma neuronal-derived exosomes (Goetzl *et al.*, 2016). Likewise, CSF levels of GAP43 were found to be altered in separate studies in PD (Sjogren *et al.*, 2000) and in Alzheimer`s disease (Sjogren *et al.*, 2001, Goetzl *et al.*, 2016). Our observation of GRIA3 and its correlation with cognitive impairment supports previous observations of reductions of AMPA receptors trafficking, or anchoring into dendritic spines with synaptic and cognitive disturbances (Henley and Wilkinson, 2013). Interestingly, significant cognitive associations with GRIA3 were present only in dementia with Lewy body and Parkinson`s disease dementia with no apparent association found in Alzheimer`s disease. The marked reductions of synaptic proteins in dementia with Lewy body and Parkinson`s disease dementia patients could reflect a greater frontal degeneration in LBD in comparison with Alzheimer`s disease, which usually affects the prefrontal cortex less than other medial and lateral temporal areas (Burton *et al.*, 2012). Together with previous findings showing alterations in levels of strategic synaptic proteins such as Rab3A, PSD95 and SNARE proteins, and their correlation to cognitive domains (Gottschall *et al.*, 2010, Mukaetova-Ladinska *et al.*, 2013, Howlett *et al.*, 2014, Vallortigara *et al.*, 2014, Whitfield *et al.*, 2014), our results provide support to the link between cognitive performance and synaptic protein loss in LBD. In line with our previous study, the current work confirms the power of synaptic proteins (Bereczki *et al.*, 2016) in discriminating patients with neurodegenerative diseases from controls with good sensitivity and specificity (>80%). In addition, we also found that GAP43 together with SNAP47 contributed to a clear separation between Parkinson`s disease dementia and Alzheimer`s disease patients, highlighting the potential role of these proteins in disease discrimination.

Alpha-synuclein is deeply involved in the synaptic vesicle trafficking required for a proper neurotransmitter release (Sidhu *et al.*, 2004). Although we selected Alzheimer`s disease cases with

low alpha-synuclein pathology, we did not observe any difference in the overall levels of monomeric alpha-synuclein protein neither between Parkinson`s disease dementia and Alzheimer`s disease nor between dementia with Lewy body and Alzheimer`s disease which could be partly due to the relative vulnerability of alpha-synuclein to post mortem delay and storage temperature (Ferrer *et al.*, 2007). This finding is in agreement with a previous proteomics study (Shi *et al.*, 2009) carried out in PD patients while another proteomics study in patients with Parkinsonism-dementia complex of Guam reported accumulation of alpha- synuclein levels (Yang *et al.*, 2007). The occasional correlations observed between synaptic proteins and alpha-synuclein scores in Parkinson`s disease dementia and dementia with Lewy body indicate that there is a potential association but more evidence is needed.

There are some limitations related to the current study. Although we were able to validate our findings from the proteomic comparison study it is possible that some important synaptic protein level changes may have been missed due to the relatively small number of patients in the LC-MS analyses, or due to post mortem delay times. Due to the exploratory nature and the small sample size of the study, correlations with cognitive impairment are presented without adjustments for multiple comparisons; thus these findings should be interpreted with caution and require confirmation in larger samples. The Alzheimer`s disease group presented more severe cognitive impairment, with longer dementia duration time compared to dementia with Lewy body and Parkinson`s disease dementia, which may have influenced the findings. Another caveat to consider is that most patients had advanced disease and the levels of synaptic proteins may differ in the earlier stages of disease progression however, these changes are likely to start early on, which is supported by the association with cognitive impairment.

In spite of these limitations, our results suggest that synaptic proteins have an important predictive and discriminative value in neurodegenerative disorders, which needs to be explored further. Moreover, the independent validation by antibodies of the level alterations of several synaptic proteins revealed by proteomics highlights the robustness of this method. *In vivo* studies using imaging and CSF are needed to explore synaptic protein changes at early disease stages. We believe that pinpointing overall alterations of synaptic proteins occurring in dementia patients brings us one step closer to a disease-specific biological target for prevention and therapeutic strategies. We anticipate their importance as a treatment target and potential as a future biomarker of disease progression for clinical trials as the therapeutic intervention window based on synaptic repair and regeneration is considerably longer than the currently used toxin-clearance approaches.

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performed all experiments. D.A., P.T.F., T.H. and C.B. provided clinical and/or neuropathological data. J.B.P. performed multivariate statistical analyses. B.W. and J.H.B. contributed to the conceptual and experimental framework. All authors contributed to the writing of the manuscript.

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7. TABLES AND FIGURES

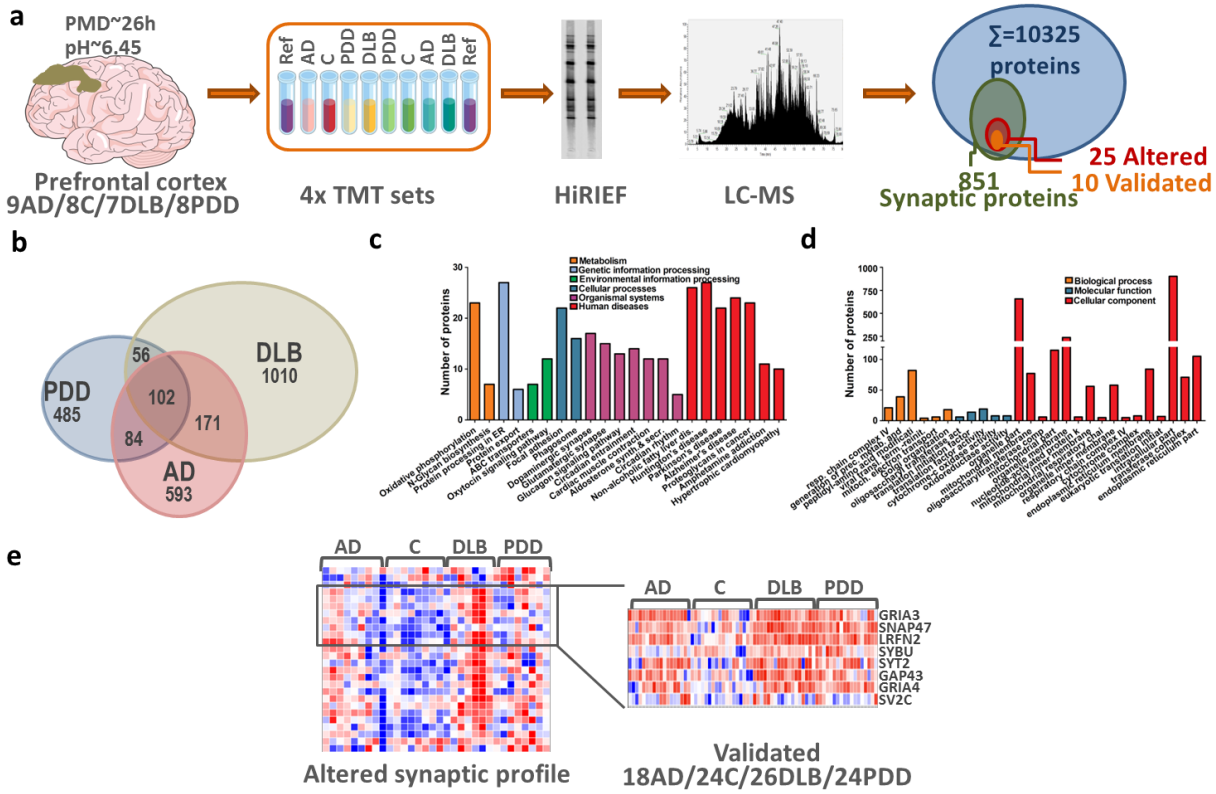

Figure 1. Proteomic data analyses. 32 post-mortem brain samples underwent proteome profile comparison. Samples were labelled at peptide level with four sets of isobaric tags (TMT10plex), each containing eight channels with randomized samples and two channels with the internal reference sample (Ref), followed by fractionation into 72 fractions by HiRIEF with the broad range IPG 3-10 strip prior to LC-MS analysis (a). Schematic representation of the number of differentially regulated proteins across disease groups (b). Differentially regulated proteins in dementia with Lewy body were further analyzed for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (c) and gene ontology (GO) terms (d). From the significantly altered synaptic proteins GRIA3, SNAP47, LRFN2, SYBU, SYT2, GAP43, GRIA4 and SV2C were chosen for

further validation with ELISA or Western blot analyses in a larger cohort (e) (2 synaptic proteins neurogranin, and CAMK2 were previously found to be altered by us within the same cohort). Abbreviations: AD, Alzheimer`s disease; C, Non-demented controls; dementia with Lewy body, dementia with Lewy bodies; PDD, Parkinson`s disease with dementia; HiRIEF, High Resolution IsoElectric Focusing; LC-MS, liquid chromatography - mass spectrometry.

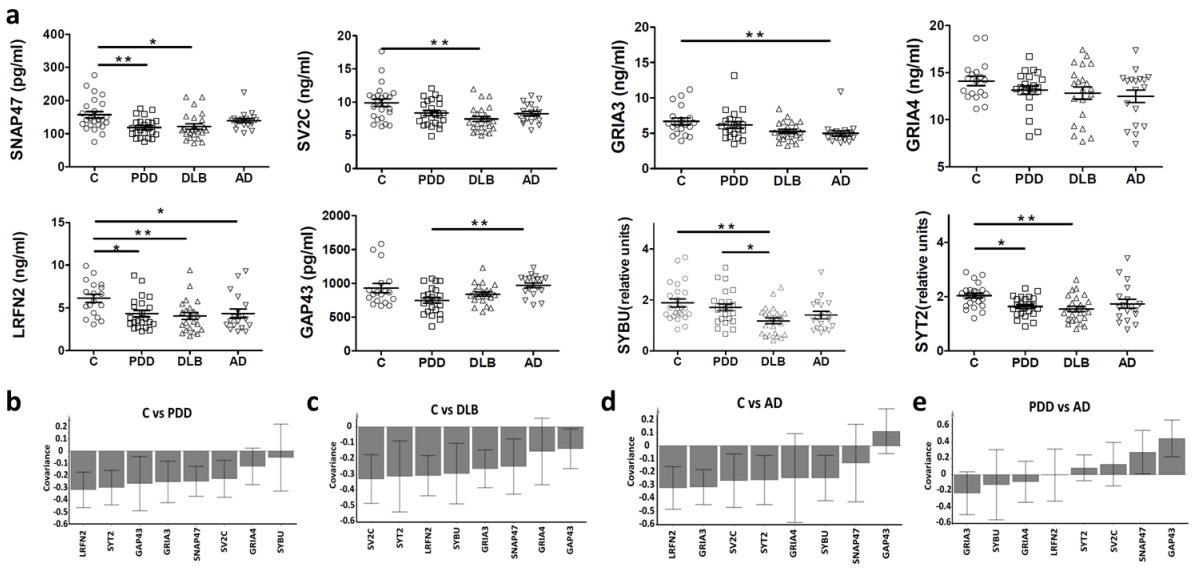

Figure 2. Changes in synaptic protein levels and their contribution to discriminating patient groups. Synaptic protein levels differed between the dementia groups (a). Univariate statistical analyses were performed using Kruskal-Wallis test followed by post hoc Dunn`s multiple comparison test. Multivariate analyses show the contribution of synaptic proteins to discriminate controls from the different patient groups (b-e). Plots showing the variables of importance and their corresponding jack-knifed confidence intervals for the separation between controls and Parkinson`s disease dementia patients (b), controls and dementia with Lewy body patients (c), controls and Alzheimer`s disease patients (d) and Parkinson`s disease dementia patients and Alzheimer`s disease Patients (e). A measure with high covariance is more likely to have an impact on group separation than a variable with low covariance. Measures with confidence intervals that include zero have low reliability.

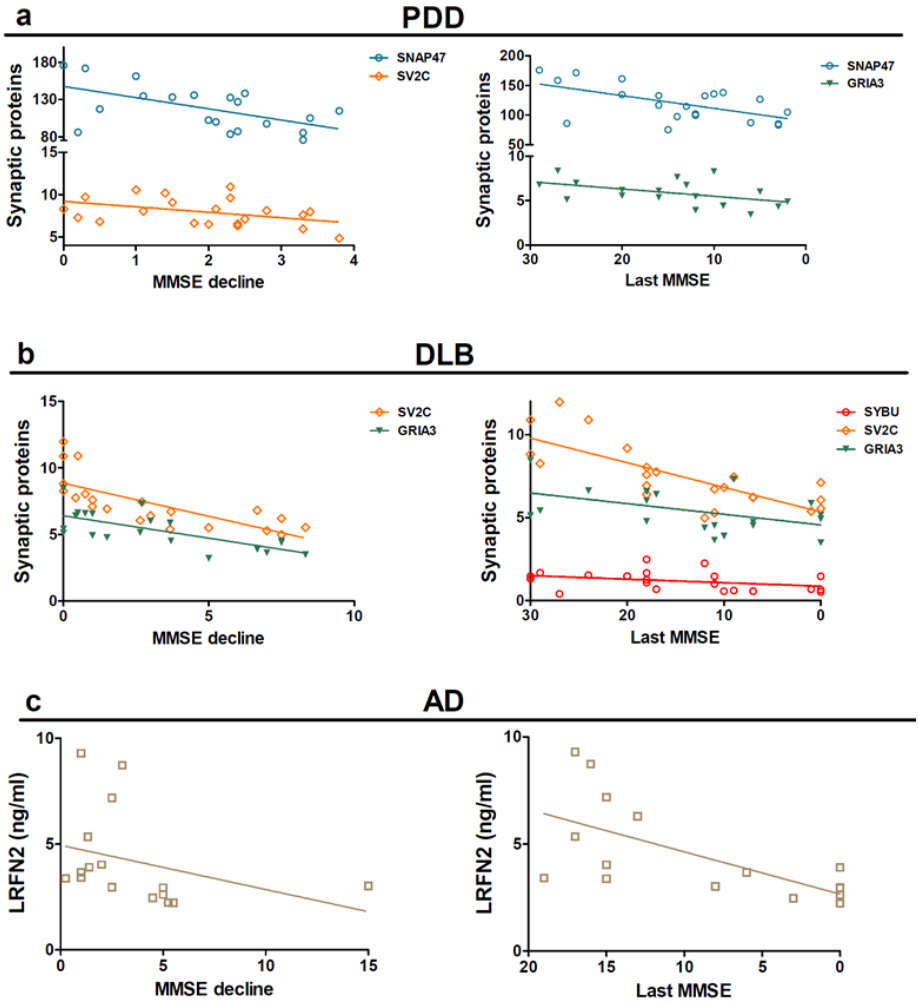

Figure 3. Correlations between synaptic proteins and cognitive impairment in Parkinson`s disease dementia (A), dementia with Lewy body (B) and Alzheimer`s disease (C). Decreased SNAP47, SV2C and GRIA3 concentrations (a) correlated with cognitive impairment in Parkinson`s disease dementia. SV2C and GRIA3 concentrations are negatively correlated with the rate of MMSE decline in dementia with Lewy body (b) showing, along with SYBU levels, positive correlations with the last MMSE scores (b). Negative correlations between LRFN2 concentrations and the rate of MMSE decline as well as positive correlations with the last MMSE scores were observed in Alzheimer`s disease (c). Associations were analysed using Spearman correlations.

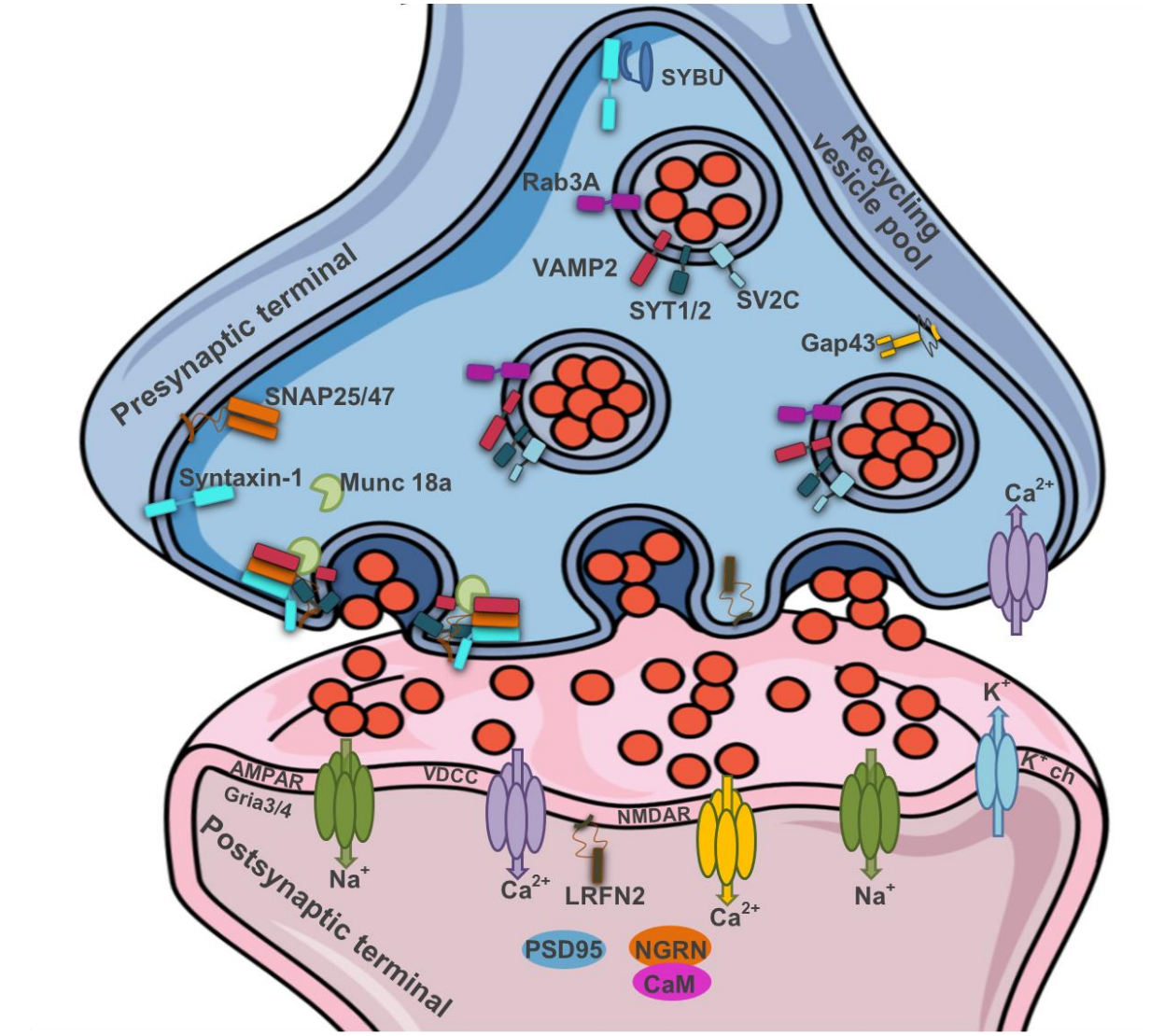

Figure 4. Schematic overview of synaptic proteins with altered levels in dementia. The diagram depicts the proteins involved in the synaptic vesicle cycle focusing on the docking and priming proteins (VAMP2, Syntaxin-1, SNAP, Munc18a), along with proteins involved in the recycling of synaptic vesicles (Rab3A, SV2C) as well as postsynaptic proteins (NRGN, PSD95, LRFN2) and receptor proteins (GRIA3, 4) found to be differentially regulated in the various dementias. Abbreviations used: NRGN, neurogranin; GAP43, neuromodulin; AMPAR, AMPA receptors/GRIA; NMDAR, N-Methyl-D-aspartic acid receptors; VDCC, voltage-dependent calcium channel.

Table 1. Demographics and clinical characteristics of the subjects included in this study

	C		PDD		DLB		AD	
	MS (N=8)	**ELISA (N=24)**	**MS (N=8)**	**ELISA (N=24)**	**MS (N=7)**	**ELISA (N=26)**	**MS (N=9)**	**ELISA (N=18)**
Age (Mean ±SD)	83 ±3.8	80.2 ±7.5	82.5 ±6	81.8 ±4.8	83.38 ±3.5	81.1 ±6.5	85.67 ±2.7	88.1 ±7.3
Gender	4M/4F	14M/10F	3M/5F	10M/14F	3M/4F	17M/9F	3M/6F	6M/12F
PMD (h) (Mean±SD)	35.2 ±18.4	38.8 ±23.4	25.3 ±7.8	33.9 ±15.8	21.7 ±12.1	27.6 ±21.3	23.7 ±10	35.0 ±22.8
pH (Mean ±SD)	6.45 ±0.3	6.46 ±0.3	6.61 ±0.3	6.53 ±0.3	6.45 ±0.5	6.43 ±0.4	6.28 ±0.3	6.30 ±0.3
Years of dementia	-	-	3.8 ±2.5	2.78 ±2	4 ±1.5	5.9 ±3	9.1 ±2.1	9.7 ±2.8
Hoehn&Yahr scale	-	-	4.75 ±0.46	4.57 ±1.08	NA	NA	-	-
Last MMSE	NA	NA	12.6 ±7.7	14.1 ±8.0	22 ±9.1	14.4 ±9.8	7.67 ±7.8	8.5 ±7.6
MMSE decline (y)	NA	NA	2.1 ±1.2	1.8 ±1.2	2.5 ±3.3	2.9 ±2.8	4.2 ±4.5	3.5 ±3.5
Aβ plaque	0.37 ±0.7	0.36 ±0.65	1.85 ±1.3	1.3 ±1.2	1.7 ±1.4	1.96 ±1.0	2.88 ±0.3	2.72 ±0.7
Tangle	0	0.18 ±0.4	0.33 ±0.5	0.43 ±0.5	0.7 ±0.5	0.85 ±0.7	2.67 ±0.5	2.5 ±0.6
α-synuclein	-	-	0.7 ±1.1	0.6 ±0.8	2.3 ±0.9	1.8 ±1.1	0.22 ± 0.4	0.18 ±0.4

Mean values are shown followed by standard deviation. One-way ANOVA followed by Bonferroni post-hoc tests showed Alzheimer`s disease patients were older compared to the other diagnostic groups (ANOVA, $F_{(3,91)}$=5.791, p=0.001 in control; p=0.019 in Parkinson`s disease dementia; p=0.05 in dementia with Lewy body). Dementia with Lewy body and Alzheimer`s disease had longer dementia duration than Parkinson`s disease dementia (ANOVA, $F_{(2,44)}$=26.738 p=0.006 Parkinson`s disease dementia versus dementia with Lewy body; p=<0.001 Parkinson`s disease dementia versus Alzheimer`s disease; p=0.002 dementia with Lewy body versus Alzheimer`s disease). There were no significant differences between diagnostic groups in other variables except cognition and pathology, which is further discussed in Fig. 3 and Supplementary Tables 4 and 5. Abbreviations: PMD, post-mortem delay; MMSE, mini-mental state examination; MS, mass spectrometry; C control; PDD, Parkinson`s disease with dementia; DLB, dementia with Lewy body; AD, Alzheimer`s disease.

Table 2. List of synaptic proteins differentially expressed between dementia cases and controls based on MS data analysis.

	Gene ID	Uniprot ID	Protein name	Fold change (min-max)	q value (%)	min. pep tides	min. quant. PSMs
PDD	**SV2C**	**Q496J9**	**Synaptic vesicle glycoprotein 2C**	**0.6 (1.7-0.3)**	**12**	**3**	**5**
	NRGN§#	**Q92686**	**Neurogranin**	**0.42 (1.5-0.2)**	**0**	**1**	**5**
	CBLN4§	Q9NTU7	Cerebellin-4	0.7 (0.9-0.5)	0	1	1
	BDNF§	P23560	Brain-derived neurotrophic factor	0.73 (1.0-0.6)	0	1	1
	GAP43§	**P17677**	**Neuromodulin**	**0.74 (1.1-0.6)**	**0**	**57**	**714**
DLB	**GRIA3**	**P42263**	**Glutamate receptor 3**	**0.56 (1.3-0.3)**	**3.5**	**7**	**9**
	CAMK2A#	**Q9UQM7**	**Calcium/calmodulin-dependent protein kinase type II subunit alpha**	**0.6 (1.4-0.4)**	**3.5**	**24**	**100**
	SYBU	**Q9NX95**	**Syntabulin**	**0.61 (1.1-0.4)**	**3.5**	**2**	**2**
	VDAC2	P45880	Voltage-dependent anion-selective channel protein 2	0.62 (1.6-0.4)	3.5	14	90
	ARC	Q7LC44	Activity-regulated cytoskeleton-associated protein	0.62 (1.0-0.4)	3.5	1	1
	RAB11A	P62491	Ras-related protein Rab-11A	0.63 (1.0-0.4)	3.5	1	1
	PDYN	P01213	Proenkephalin-B	0.64 (1.3-0.4)	3.5	1	1
	GRIA4	**P48058**	**Glutamate receptor 4**	**0.64 (1.3-0.3)**	**3.5**	**2**	**2**
	SYT2	**Q8N9I0**	**Synaptotagmin-2**	**0.64 (1.4-0.4)**	**3.5**	**7**	**9**
	CAMK2G	Q13555	Calcium/calmodulin-dependent protein kinase type II subunit gamma	0.64 (1.4-0.5)	3.5	19	32
	CNIH2	Q6PI25	Protein cornichon homolog 2	0.65 (1.3-0.4)	3.5	1	1
	KCNIP2	Q9NS61	Kv channel-interacting protein 2	0.65 (1.4-0.4)	3.5	1	1
	SNAP47	**Q5SQN1**	**Synaptosomal-associated protein 47**	**0.66 (0.9-0.4)**	**3.5**	**2**	**2**
	TECR	Q9NZ01	Very-long-chain enoyl-CoA reductase	0.67 (1.2-0.4)	3.5	5	8
	CACNG2	Q9Y698	Voltage-dependent calcium channel gamma-2 subunit	0.67 (1.0-0.5)	3.5	2	2
	PVRL3	Q9NQS3	Nectin-3	0.68 (1.1-0.4)	3.5	1	1
	LRFN2	**Q9ULH4**	**Leucine-rich repeat and fibronectin type-III domain-containing protein 2**	**0.68 (1.1-0.5)**	**3.5**	**3**	**3**
	GRIK2	Q13002	Glutamate receptor ionotropic, kainate 2	0.68 (1.2-0.5)	3.5	2	2
	CACNG3	O60359	Voltage-dependent calcium channel gamma-3 subunit	0.7 (1.0-0.5)	3.5	1	1
	TNK2	Q07912	Activated CDC42 kinase 1	0.71 (1.1-0.5)	3.5	2	2
	CAMKK1	Q8N5S9	Calcium/calmodulin-dependent protein kinase kinase 1	0.73 (1.1-0.6)	3.5	12	18

Differences were assessed with respect to the control group. The exceptions are denoted by §, which refers to differences between Parkinson`s disease dementia and Alzheimer`s disease. Proteins in bold were chosen for further analyses with ELISA or Western blot. # Denotes proteins previously measured in this cohort, which are discussed in the results and discussion sections. In addition to fold change, including minimum and maximum fold change values in parenthesis, q-value, the minimal number of unique peptides and the minimal number of quantified PSMs per TMT set is shown.

Table 3. Differences in synaptic protein levels between control and dementia groups using WB and ELISA data

	C (N=24)	**PDD (N=24)**		**DLB (N=25)**		**AD (N=18)**	
SNAP47(pg/mL)	156.9 ±48.6	**118.8 ±29.2**	**p=.005**	**121.9 ±39.1**	**p=.022**	139.5 ±26.5	p=.848
SV2C (ng/mL)	9.9 ±2.7	8.3 ±1.9	p=0.068	**7.5 ±1.9**	**p=.01**	8.3 ±1.5	p=.085
GRIA3 (ng/mL)	6.68 ±2.1	6.16 ±2.1	p=1.00	5.3 ±1.3	p=.109	**4.9 ±1.6**	**p=.036**
GRIA4 (ng/mL)	14.1±2.1	13.1±2.0	p=1.00	12.8±2.9	p=.721	12.5±2.8	p=.358
LRNF2 (ng/mL)	6.1±2	**4.3±1.8**	**p=.032**	**4.1±1.9**	**p=.01**	**4.3±2.2**	**p=.05**
GAP43 (pg/mL)	931.5±282	**747.4±202**	**#p=.004**	842.8±148	p=1.00	972.8±156	p=1.00
SYBU	1.88±0.75	1.71±0.67	p=1.00	**1.18±0.54**	**p=.002, §p=.033**	1.4±0.61	p=.799
SYT2	2.03±0.43	**1.64±0.36**	**p=.037**	**1.55±0.48**	**p=.006**	1.74±0.70	p=.330

Differences in protein levels between disease groups and controls were determined using Kruskal–Wallis test followed by Dunn's post hoc test. ELISA values are expressed in pg/mL or ng/mL (means ±standard deviation). Western blot changes are expressed in relative units. p values represent statistically significant differences between dementia and non-demented control groups. # Denotes significant differences between Parkinson`s disease dementia and Alzheimer`s disease groups. § Denotes significant differences between dementia with Lewy body and Parkinson`s disease dementia groups.

Table 4. Sensitivity, specificity, positive and negative predictive values for each model

Models	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
C vs PDD	73.7 (48.8-90.9)	73.9 (51.6-89.8)	70.0 (45.7-88.1)	77.3 (54.6-92.2)
C vs DLB	83.3 (62.6-95.3)	80.0 (59.3-93.2)	80 (59.3-93.2)	83.3 (62.6-95.3)
C vs AD	81.3 (54.4-95.6)	80.8 (60.7-93.5)	72.2 (46.5-90.3)	87.5 (67.6-97.3)
PDD vs AD	77.8 (52.4-93.6)	80.0 (56.3-94-3)	77.8 (52.4-93.6)	80.0 (56.3-94.3)

C, controls; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.