

Targeting Synaptic Pathology with a Novel Affinity Mass Spectrometry Approach^{*§}

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We report a novel strategy for studying synaptic pathology by concurrently measuring levels of four SNARE complex proteins from individual brain tissue samples. This method combines affinity purification and mass spectrometry and can be applied directly for studies of SNARE complex proteins in multiple species or modified to target other key elements in neuronal function. We use the technique to demonstrate altered levels of presynaptic proteins in Alzheimer disease patients and prion-infected mice. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.M114.040113, 2584–2592, 2014.

One prominent pathological feature of neuropsychiatric disorders such as Alzheimer disease (AD)¹ is severe synaptic loss (1–3). Previous reports of AD patients have shown that presynaptic dysfunction might occur early in the disease process (1, 4). Cortical synapse pathology has also been shown to correlate to the severity of dementia more closely than other pathological hallmarks of AD such as plaques and neurofibrillary tangles (5, 6). The SNARE proteins are essential components for the regulation of neurotransmitter exocytosis at the presynaptic site (7). Animal models suggest that changed expression or modification of SNARE complex proteins (synaptosomal-associated protein 25 (SNAP-25), syn-

taxin-1, and vesicle-associated membrane protein (VAMP)) alters synaptic function and is an interesting target for the development of therapeutics for neuropsychiatric illness (8, 9). The constituents of the SNARE complex are either localized in synaptic vesicles (VAMPs) or anchored at the presynaptic plasma membrane (SNAP-25 and syntaxin). The SNARE proteins are tightly assembled, and subsequent neurotransmitter release of the complex is quickly dissociated by *N*-ethylmaleimide-sensitive factor (7, 10–12). Because they are both strongly associated into complexes and membrane associated, the SNARE proteins are difficult to analyze via mass spectrometry, which is incompatible with most detergents necessary for the solubilization of proteins. Each SNARE complex protein exists in several isoforms that are differently distributed within the central nervous system (13–18). Post-translational modifications and truncated variants of the SNARE proteins make investigation of the protein expression even more complicated.

In this study we developed an approach for the characterization and concurrent quantification of SNARE complex proteins that combines affinity purification by immunoprecipitation and mass spectrometry (IP-MS). We used precipitation with monoclonal antibodies against SNAP-25 to target the SNARE complex proteins and nanoflow LC–tandem mass spectrometry (LC-MS/MS) to characterize the co-immunoprecipitated interaction partners. Selected reaction monitoring (SRM) on a triple quadrupole mass spectrometer coupled to a microflow LC system was used for quantification of the SNARE proteins. To demonstrate the usability of the IP-MS method, we performed a comparison of SNARE complex protein levels in brain tissue from AD patients and age-matched controls, as well as a study of SNARE complex protein levels in brain tissue from prion-infected mice.

EXPERIMENTAL PROCEDURES

Human Brain Tissue Samples—This study involved autopsy-confirmed patients with AD ($n = 15$) and age-matched controls ($n = 15$). Brain tissues from the superior parietal gyrus were analyzed. All brain tissues were obtained from the Netherlands Brain Bank. Braak and Braak criteria, which are based on the distribution of neurofibrillary tangles, were used to categorize the stage of AD (19). All AD patients were classified as Braak stage 5 or 6, and the controls were Braak stage 0 or 1. [Supplemental Table S1](#) shows the clinical and demographic characteristics of the groups.

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Received April 11, 2014, and in revised form, June 12, 2014

Published, MCP Papers in Press, June 27, 2014, DOI 10.1074/mcp.M114.040113

Author contributions: A.B., H.Z., K.B., and A.O. designed research; A.B. and A.O. performed research; A.B., G.B., W.G.H., J.A.M., J.J., G.R.M., and A.O. contributed new reagents or analytic tools; A.B., G.B., W.G.H., and A.O. analyzed data; A.B., G.B., W.G.H., J.A.M., J.J., G.R.M., H.Z., K.B., and A.O. wrote the paper.

¹ The abbreviations used are: AD, Alzheimer disease; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP-25, synaptosomal-associated protein 25; VAMP, vesicle-associated membrane protein; IP-MS, immunoprecipitation–mass spectrometry; SRM, selected reaction monitoring; w.p.i., weeks post-infection; FA, formic acid.

Mouse Brain Tissue Samples—All animal work conformed to UK regulations and institutional guidelines and was performed under Home Office guidelines. tg37 (20) were inoculated with 1% brain homogenate of Chandler/Rocky Mountain Laboratory prions or with normal brain homogenate aged 3 to 4 weeks, as described in Ref. 21. Hippocampi were processed at 6, 7, 8, 9, and 10 weeks post-infection (w.p.i.) and stored at -80°C prior to homogenization. For all analyses, $n = 3$ mice unless otherwise stated.

Homogenization of Brain Tissue—The brain extraction procedure was performed as described by Öhrfelt *et al.*, with minor modifications (22). Briefly, 100 ± 10 mg of brain tissue was homogenized on ice in 1 ml of Tris-buffer (10 mM Tris-HCl, pH 6.8) containing complete protease inhibitor (Roche Diagnostics GmbH). Centrifugation of the homogenate occurred at $31,000g$ for 1 h at $+4^{\circ}\text{C}$, and then the supernatant was collected (Tris). One milliliter of Tris-buffer containing 0.5% Triton X-100 (Union Carbide Corporation, Danbury, CT) containing complete protease inhibitor was added to the pellet, which was then homogenized on ice and sonicated using a micro-probe sonicator. The centrifugation step was repeated, and the supernatant was collected (0.5% Triton (*i.e.* membrane-bound fraction)). The same procedure was repeated with the addition of Tris-buffer containing 2% Triton and complete protease inhibitor and with the addition of Tris-buffer containing 0.5% SDS and complete protease inhibitor for a final centrifugation at $+12^{\circ}\text{C}$ (SDS fraction (*i.e.* membrane-raft associated fraction)). All supernatants were aliquoted and stored at -80°C pending analysis. For protein quantitation, Protein DC assay (Bio-Rad Laboratories) reagent was used. This reagent is a reducing agent and is detergent compatible.

Antibodies and Recombinant Protein of SNAP-25—The following antibodies were used: mouse monoclonal antibody SP12 recognizing SNAP-25 (23), mouse monoclonal antibody SMI81 (Covance, Princeton, NJ) against SNAP-25 (24), and a polyclonal anti-SNAP-25 antibody raised in rabbit according to Ref. 25. Recombinant standard protein of SNAP-25 was purchased from Origene (Rockville, MD).

Immunoprecipitation—The immunoprecipitation of brain tissue extracts was performed according to Ref. 22, with minor modifications. Briefly, an aliquot (1 μg) of the mouse monoclonal antibody SP12 (1 g/l), the mouse monoclonal antibody SMI81 (1 g/l), or IgG from murine serum (1 g/l, Sigma-Aldrich) (a negative control) was separately added to 100 μl of magnetic Dynabead M-280 Sheep anti-mouse IgG (Invitrogen) and incubated for 1 h on a rocking platform at room temperature. The beads were washed three times with 1 ml of PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4). The antibodies were cross-linked using 20 mM dimethyl pimelimidate dihydrochloride (Sigma-Aldrich) and 0.2 M triethanolamine (pH 8.2; Sigma-Aldrich) according to the manufacturer's product description. The cross-linked beads were washed two times in PBS and blocked with Roti-Block (Carl Roth, GmbH Karlsruhe, Germany) for 1 h on a rocking platform at room temperature. Each brain tissue extract (0.5% Triton and SDS) was adjusted with 20% Triton and PBS to a final concentration of 0.2% Triton and a final concentration of 26 μg of total protein. Samples and magnetic beads were incubated overnight on a rocking platform at $+4^{\circ}\text{C}$. The magnetic bead-sample solution was transferred to a KingFisher magnetic particle processor (Thermo Fisher Scientific) (tube 1). The following three wash steps (tubes 2–4) were conducted for 10 s in 1 ml of each washing buffer: tube 2, 0.025% Tween 20 in PBS; tube 3, PBS; and tube 4, 50 mM ammonium hydrogen carbonate (NH_4HCO_3 , pH 8.0). Then, SNAP-25 and closely interacting proteins (*e.g.* SNARE complex proteins) were eluted from the beads with 100 μl of 0.5% formic acid (FA) (tube 5) for 4 min. The eluted fractions were transferred to 0.5-ml Protein LoBind tubes (Eppendorf AG, Hamburg, Germany) and dried in a vacuum centrifuge.

Protein Digestion—The dried samples were dissolved in 10 μl of 0.1% RapiGest SF Surfactant (Waters, Milford, MA) in 50 mM NH_4HCO_3 (pH 8.0) for 1 h at room temperature. Disulfide bonds were reduced by the addition of 10 μl of 10 mM DTT (Sigma-Aldrich, St. Louis, MO) in 50 mM NH_4HCO_3 and incubation for 3 min at $+90^{\circ}\text{C}$. After cooling to room temperature, 5 μl of 10 mM iodoacetamide (Sigma-Aldrich) in 50 mM NH_4HCO_3 was added, and the samples were incubated in the dark at room temperature for 30 min. Digestion was carried out by the addition of 5 μl of trypsin solution (20 μg of Sequencing Grade Modified Trypsin (Promega, Madison, WI) dissolved in 0.01% aqueous HCl (0.1g/l) and diluted 1 to 20 in 50 mM NH_4HCO_3) and overnight incubation at $+37^{\circ}\text{C}$. To reduce the amount of RapiGest SF Surfactant in the samples, 2 μl of 10% aqueous TFA was added (resulting in pH < 2). The samples were incubated for 45 min at $+37^{\circ}\text{C}$ and then centrifuged ($16,900g$, 10 min, $+4^{\circ}\text{C}$). Twenty-five microliters of the resulting supernatants of each sample was carefully transferred to 0.5-ml Protein LoBind tubes (Eppendorf AG).

Addition of Heavy-isotope-labeled Peptide Standards—C-terminal isotopically labeled peptides containing U-13C6, U-15N4-arginine or U-13C6, U-15N2-lysine were supplied by Sigma-Aldrich at over 95% peptide purity as determined via reverse phase HPLC. Three labeled peptides were chosen as reference standards for SNAP-25 (amino acids 18–30, ADQLADESLEST[R], common for both SNAP-25A and SNAP-25B), syntaxin-1A (amino acids 95–108, SIEQSIEQEEGLN[R]), and syntaxin-1B (amino acids 94–107, AIEQSIEQEEGLN[R]). Heavy-isotope-labeled AQUATM Peptide standard for VAMP-2/3 (AQ0256, AQUATM Peptide ADALQAGASQFETSAA[K]) was also purchased (Sigma-Aldrich). The three custom synthesized peptides were dissolved and diluted in 0.1% aqueous FA and mixed to a final concentration of 5 fmol/ μl (SNAP-25, syntaxin-1B) or 10 fmol/ μl (syntaxin-1A). One vial of AQ0256 was dissolved in 100 μl of 20% acetonitrile in 0.1% aqueous FA, diluted in 0.1% aqueous FA, and mixed with the rest of the heavy-isotope-labeled peptides to a final concentration of 5 fmol/ μl . A 25- μl aliquot of the reference peptide mixture was added to each immunoprecipitated brain homogenate sample after digestion and centrifugation.

LC-MS/MS Analysis of Digested SNARE Complex Proteins—Aliquots (25 μl) of the 1:1 mixtures of stable-isotope-labeled standard peptides and immunoprecipitated SNARE proteins were transferred to LC vials (Waters) and analyzed via LC-MS/MS. The LC-MS/MS spectra were acquired with a hybrid linear quadrupole ion trap Fourier transform ion cyclotron resonance mass spectrometer equipped with a 7 T magnet (LTQ FT Ultra, Thermo Fisher Scientific, Bremen, Germany) coupled to a multidimensional nanoflow chromatography system (Ettan MDLC, GE Healthcare). A Zorbax 300 SB-C18 trap column (length, 5 mm; inner diameter, 0.3 mm; particle size, 5 μm ; Agilent Technologies, Palo Alto, CA) was used for on-line desalting, and a reversed phase Zorbax 300 SBC18 column (length, 150 mm; inner diameter, 0.075 mm; particle size, 3.5 μm ; Agilent Technologies) was used for high-resolution separation. Mobile phases were 0.1% aqueous FA (v/v) (A) and 0.1% FA in 84% acetonitrile in water (v/v) (B). The separation was performed at a flow rate of ~ 250 nL/min by applying a linear gradient of 0% to 60% B for 50 min. The LTQ FT Ultra was set to acquire positive ions and operated in the data-dependent mode, where a scan cycle consisted of one full scan mass spectrum (m/z 350–1500) acquired in the Fourier transform ion cyclotron resonance mode (resolution 25,000) followed by 10 tandem mass spectrometry (MS/MS) scans acquired in linear quadrupole ion trap mode using collision-induced dissociation and wideband activation. Dynamic exclusion was enabled with a repeat count of 2 and an exclusion duration of 120 s. The MS/MS isolation width was set to 3 m/z units, and the normalized collision energy to 35. Each scan consisted of three microscans.

Database Search and Bioinformatic Analysis—Database searches were submitted to the in-house database server by using Mascot Deamon 2.3.0 (Matrix Science, London, UK). Database search parameters were database (UniProtKB Human 131030, 88,266 sequences, 35,040,462 residues), taxonomy (*Homo sapiens*), enzyme (trypsin), variable modifications (acetyl (N-terminal), oxidation (M), Label:13C(6)15N(2) (K), and Label:13C(6)15N(4) (R)), fixed modifications (carbamidomethyl (C)), mass values (monoisotopic), peptide mass tolerance (5 ppm), fragment mass tolerance (0.5 Da), maximum number of missed cleavages (two), and instrument type (electrospray ionization trap). The Mascot result files were exported as XML files with ion score cutoff (1), threshold type (identity), protein scoring (MudPIT), same-set protein hits (additional proteins that span the same set of peptides) included, and bold red required. The Mascot XML files were imported into the ProteinCenter software package (version 3.8.2014, Thermo Fisher Scientific). In ProteinCenter, the datasets from each sample were filtered by removing first peptides with individual ion scores less than or equal to 31 (where a score >31 indicated identity or extensive homology ($p < 0.05$)) and subsequently proteins with less than two unique peptides. All datasets from the same biochemical fraction were then compared and clustered on indistinguishable proteins (possible peptide assignments not included). To generate the list of proteins specific to immunoprecipitation with a specific antibody, all proteins that were present in the negative controls were removed. To represent each cluster of indistinguishable proteins, the protein was chosen that was present in most datasets or, if that was the same, that had the strongest evidence of existence stated in UniProtKB. In the case that that was the same, all proteins were stated on the same row.

Quantification of High-mass-accuracy Precursor Ions—Peak detection and integration were performed using DeCyder 2.0 (GE Healthcare) using processing parameters as described in Ref. 26. In-house-developed software (Sequence and PeakExtractor) was used for *in silico* digestion and automatic peak mass matching.

SRM-MS—Aliquots (25 μ l) of the 1:1 mixtures of stable-isotope-labeled standard peptides and immunoprecipitated SNARE proteins were transferred to LC vials (SUN-SRi) and analyzed via SRM-MS using an Accela 1250 pump (Thermo Fisher Scientific) coupled to a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific) with an IonMax source and HESI-II electrospray probe equipped with a high-flow metal needle (Thermo Fisher Scientific). Mobile phases were 0.1% aqueous FA (v/v) (A) and 0.1% FA in 84% acetonitrile in water (v/v) (B). Samples were loaded directly onto a Hypersil Gold-C18 column (length, 50 mm; inner diameter, 2.1 mm; particle size, 5 μ m; Thermo Fisher Scientific) with 0.1% aqueous FA at 200 μ l/min. After 2 min of loading, the peptides were eluted off the column using the following linear gradient steps: 4 min 17% B; 12 min 23% B; 15 min 100% B. The following global MS parameters were used: positive ion mode; spray voltage, 3.5 kV; vaporizer temperature, 350 °C; sheath gas pressure, 40 psi; auxiliary gas pressure, 25 (arbitrary units); capillary temperature, 350 °C; collision gas pressure, 1.9 mTorr. Pinpoint software version 1.1 (Thermo Fisher Scientific) was used for method optimization and data processing.

Sandwich ELISA Methods for SNAP-25—A combination of mouse monoclonal antibody SP12 or mouse monoclonal antibody SMI81, as a capturing antibody, and a polyclonal anti-SNAP-25 antibody, as a detection antibody, resulted in the most sensitive detection method for SNAP-25 in human brain tissue extract. Maxisorp plates (Nunc) were coated with 100 μ l of mouse monoclonal antibody SP12 (1 g/l) or mouse monoclonal antibody SMI81 (1 g/l), diluted 1:1000 in 50 mM carbonate buffer, pH 9.6, and incubated overnight at +4 °C. The plates were washed four times with 350 μ l of PBS-T (0.05% v/v Tween-20 in PBS) and blocked with 200 μ l of Roti-Block (Carl Roth) (diluted with PBS-T) for 1 h at room temperature. The plates were

washed four times with 350 μ l of PBS-T. The washing procedure was repeated between all following incubation steps. All samples were analyzed in duplicate. Brain tissue extracts were diluted 1:500 (SP12 and SMI81, Triton) and 1:1500 (SP12 and SMI81, SDS) in 0.1 \times Rotiblock, 0.01% Triton X-100 in PBS-T (diluent buffer). The standard of SNAP-25 was diluted in diluent buffer to a concentration range of 31.3–8000 mg/l. Samples (100 μ l) of diluted brain tissue extract and standard were incubated for 1 h on a plate shaker at room temperature. Then, 100 μ l of polyclonal anti-SNAP-25 antibody was diluted in diluent buffer to a final concentration of 0.5 mg/l, and the plates were incubated for 1 h on a plate shaker at room temperature. Thereafter, 100 μ l of anti-rabbit IgG-biotin antibody (Sigma-Aldrich) was diluted 1:100,000 in diluent buffer and incubated for 1 h on a plate shaker at room temperature. NeutrAvidin Horseradish Peroxidase conjugate (1 g/l) (Thermo Fisher Scientific) diluted 1:10,000 in dilution buffer was incubated for 1 h on a plate shaker at room temperature, and development occurred with the addition of 100 μ l of 3,3',5,5'-tetramethylbenzidine substrate (TMB Peroxidase EIA Substrate Kit, Bio-Rad Laboratories). The reaction was quenched with 100 μ l of H₂SO₄ (2 M). The absorbance was measured at 450 nm. The concentration of SNAP-25 in samples of brain tissue extracts was calculated from the standard curve. Samples below the limit of detection were conservatively set to this limit. For each sample, a ratio was calculated where the SNAP-25 level was divided by the total protein concentration.

Statistical Analysis—SPSS 20.0 was employed for the statistical analyses for human samples. Because the distributions of most investigated proteins were not normal (Shapiro–Wilk test, $p < 0.05$), non-parametric statistics were used for the analysis of human brain tissue samples. Data are given as median (interquartile range). The Mann–Whitney *U* test was used to investigate group differences. The correlation coefficients (ρ) were calculated using Spearman's two-tailed correlation test. Data from the mice study are given as mean \pm S.E. Student's unpaired *t* test was applied using GraphPad Prism 5.

Ethics—The ethical principles adhered to by Netherlands Brain Bank can be found online.

RESULTS

In this study we used a hybrid approach that combined affinity purification through immunoprecipitation with a monoclonal antibody and quantitation and characterization with mass spectrometry analysis (22). Brain tissue from age-matched patients with AD ($n = 15$) and controls ($n = 15$) (supplemental Table S1) were biochemically fractionated according to solubility using non-ionic and ionic detergents at different concentrations. Immunoprecipitation with the monoclonal antibody SP12 (derived through immunization with a human synaptic immunoprecipitate (16, 23)) against the SNARE complex protein SNAP-25 was utilized to selectively purify proteins from the fractions containing membrane-bound proteins and membrane-raft-associated proteins. The affinity-purified proteins were digested with trypsin, and four stable-isotope-labeled peptide standards, representing the SNARE complex proteins (SNAP-25, syntaxin-1A, syntaxin-1B, and VAMP-2/3), were added.

Initially, high-resolution LC-MS/MS analysis was used to identify the SNARE proteins that were selectively immunoprecipitated from the different brain homogenate fractions. We found that all four SNARE complex proteins (SNAP-25, syntaxin-1A, syntaxin-1B, and VAMP-2) were present in the majority of the samples (Table I, supplemental Tables S2 and S3).

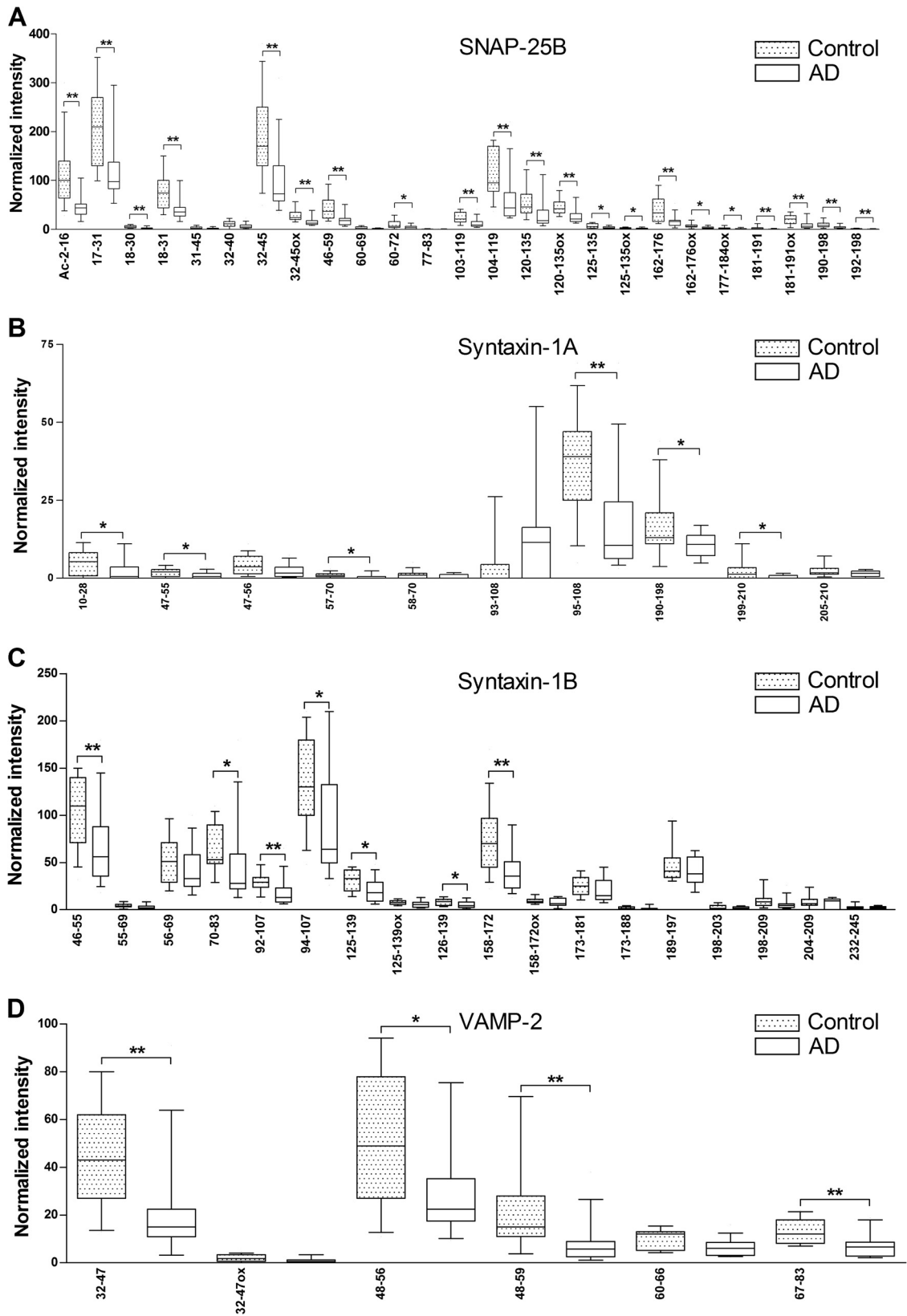
TABLE I
SNARE proteins identified with LC-MS/MS in immunoprecipitate (monoclonal antibody SP12) from membrane-bound and membrane-raft-associated protein fractions of superior parietal gyrus from controls and patients with Alzheimer disease (AD)

Protein	Fraction of samples in which the protein was found (%)		Unique peptide sequences (average)		Sequence coverage (%) (average)	
	Membrane-bound Control/AD	Membrane-raft-associated Control/AD	Membrane-bound Control/AD	Membrane-raft-associated Control/AD	Membrane-bound Control/AD	Membrane-raft-associated Control/AD
	SNAP-25B	100/100	100/100	19/15	19/16	67/63
SNAP-25A	13/0	7/7	Too few	Too few	Too few	Too few
Syntaxin-1A	100/100	100/100	9/6	9/7	24/18	28/21
Syntaxin-1B	100/100	100/100	16/15	17/15	47/45	47/44
VAMP-2	93/100	100/93	5/5	4/4	45/44	38/39
VAMP-1	73/57	87/50	4/4	3/3	30/32	24/26
VAMP-3	20/7	53/7	5/too few	4/too few	48/too few	39/too few

SNAP-25 exists in two isoforms in the brain, SNAP-25A and SNAP-25B (27). We identified SNAP-25B-specific peptides in all samples, but specific SNAP-25A peptides only in a few. This indicates that SNAP-25B levels were much higher than SNAP-25A levels, consistent with earlier findings that SNAP-25B is the predominant species in adult brain (18, 28). Overall, the identified tryptic peptides of SNAP-25B covered the whole protein (amino acids 2–198, [supplemental Fig. S1, supplemental Table S3](#)). We also found SNAP-25 to be modified by methionine excision and acetylation, which is in agreement with a previous study (24). Syntaxin-1 also exists in two isoforms (1A and 1B) (14, 29). Peptides specific for both forms were identified via MS/MS in all samples. Tryptic peptides covering most of the proteins were also detected ([supplemental Fig. S1, supplemental Table S3](#)). Concerning the VAMP/syntaxin isoforms, peptides specific for VAMP-2 and VAMP-1 were detected in most samples, and for VAMP-3 in ~20% of the samples ([supplemental Fig. S1, supplemental Table S3](#)). All detected peptides originated from the v-SNARE coiled-coil homology domains in the respective VAMP isoforms ([supplemental Fig. S1, supplemental Table S3](#)) (30). Apart from the SNARE complex proteins, several other proteins that are known or suspected to interact with the SNARE complex (e.g. complexin-1, complexin-2, syntaxin-binding protein-1, and synaptotagmin-1) were co-immunoprecipitated with SNAP-25 ([supplemental Table S2](#)) (31–33).

To estimate the SNARE protein levels, we extracted the acquired LC-MS ion chromatograms of all peptides belonging to a SNARE complex protein and compared the signal intensities between AD and control groups (Figs. 1A–1D, [supplemental Figs. S2A–S2D, supplemental Table S4](#)). We found that the intensities of most of the investigated tryptic peptides belonging to SNAP-25B, syntaxin-1B, syntaxin-1A, or VAMP-2 were significantly lower in the AD group than in the control group (Figs. 1A–1D, [supplemental Figs. S2A–S2D, supplemental Table S4](#)).

To get a more accurate quantification of the SNARE complex proteins, aliquots of immunoprecipitated (SP12) brain protein fractions from patients with AD ($n = 15$) and controls ($n = 15$) were quantified using SRM-MS. Membrane-bound fraction levels of all SNARE proteins were significantly lower in the AD group (Figs. 2A–2D, [supplemental Table S5](#)). SNAP-25 and syntaxin-1A/B levels were also significantly lower in the AD group in the membrane-raft-associated fractions (Figs. 2E–2G, [supplemental Table S5](#)). The finding of reduced SNARE protein levels in AD patients was consistent with previous studies of AD using different immunoassays (34–37). The results also demonstrate that the IP-MS method is sufficiently sensitive to detect pathological changes in SNARE protein levels in brain tissue samples from individual patients. The IP-MS levels of SNAP-25 were strongly correlated with the levels of syntaxin-1A/B and VAMP-2 (membrane-bound ($\rho = 0.886$, $p < 0.001$ for syntaxin-1A; $\rho = 0.806$, $p < 0.001$ for syntaxin-1B; and $\rho = 0.779$, $p < 0.001$ for VAMP-2) and



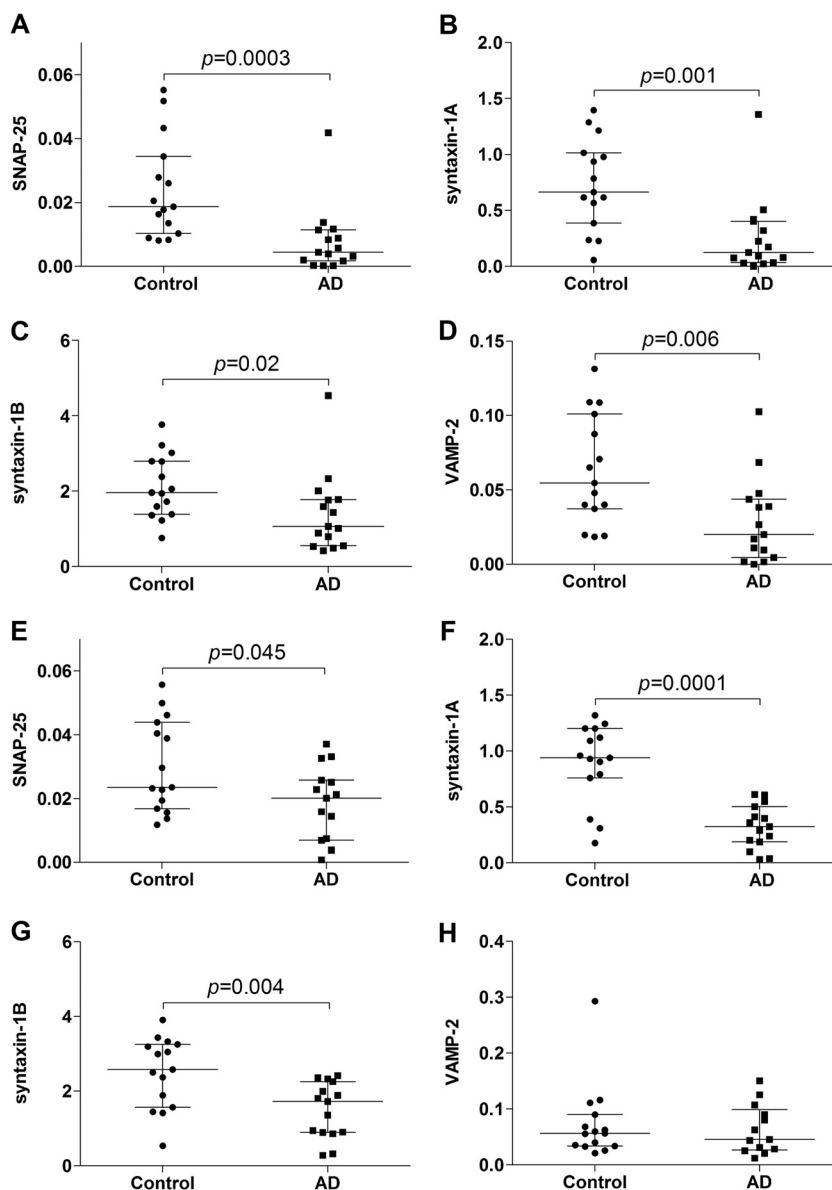


FIG. 2. Targeted mass spectrometry analyses of the SNARE complex in human brain. Individual values for the SRM-MS measured ratios (endogenous peptide/labeled peptide standard) of immunoprecipitated (SP12 antibody) SNAP-25, syntaxin-1A, syntaxin-1B, and VAMP-2/3 in biochemically fractionated membrane-bound (A–D) and membrane-raft-associated (E–H) fractions of superior parietal gyrus from controls ($n = 15$) and patients with Alzheimer disease (AD) ($n = 15$). The lower, upper, and middle lines of the error bars correspond to the 25th and 75th percentiles and medians, respectively. The Mann–Whitney U test was used to investigate group differences.

membrane-raft-associated ($\rho = 0.624$, $p < 0.001$ for syntaxin-1A; $\rho = 0.645$, $p < 0.001$ for syntaxin-1B; and $\rho = 0.513$, $p = 0.005$ for VAMP-2)). This indicates that the membrane-bound fractions of SNARE proteins are tightly associated, supporting previous work showing that the SNARE complex is stable and even resistant to the detergent SDS (10).

Potential disadvantages of the IP-MS method relative to ELISA may include lower sensitivity and reproducibility. However, when using an in-house-developed sandwich ELISA for

SNAP-25 with SMI81 (recognizes an epitope containing the N-terminally acetylated first 11 amino acids of brain SNAP-25 (24)) or SP12 as capture antibodies, we observed results similar to those of the IP-MS assay (supplemental Fig. S3). The SNAP-25 levels measured with SMI81 and SP12 ELISA were highly correlated both in the membrane-bound fractions ($\rho = 0.984$, $p < 0.001$) and in the membrane-raft-associated fractions ($\rho = 0.943$, $p < 0.001$). Furthermore, the IP-MS-measured levels of SNAP-25 were correlated with the

FIG. 1. LC-MS signal intensities of individual tryptic peptides from immunoprecipitated SNARE proteins SNAP-25B (A), syntaxin-1A (B), syntaxin-1B (C), and VAMP-2 (D) affinity-purified with the monoclonal antibody SP12 from membrane-bound fractions of superior parietal gyrus from controls and patients with Alzheimer disease (AD). Normalized intensities of tryptic peptides measured with high-resolution LC-MS, confirmed with MS/MS, and present in at least 10 controls (dotted bars) and 10 patients with AD (white bars). The lower, upper, and middle portions of the bars correspond to the 25th percentile, 75th percentile, and median, respectively. The whiskers on the bottom extend from the 10th percentile to the top 90th percentile. p values less than 0.05 and 0.01 are denoted by * and ** (Mann–Whitney U test).

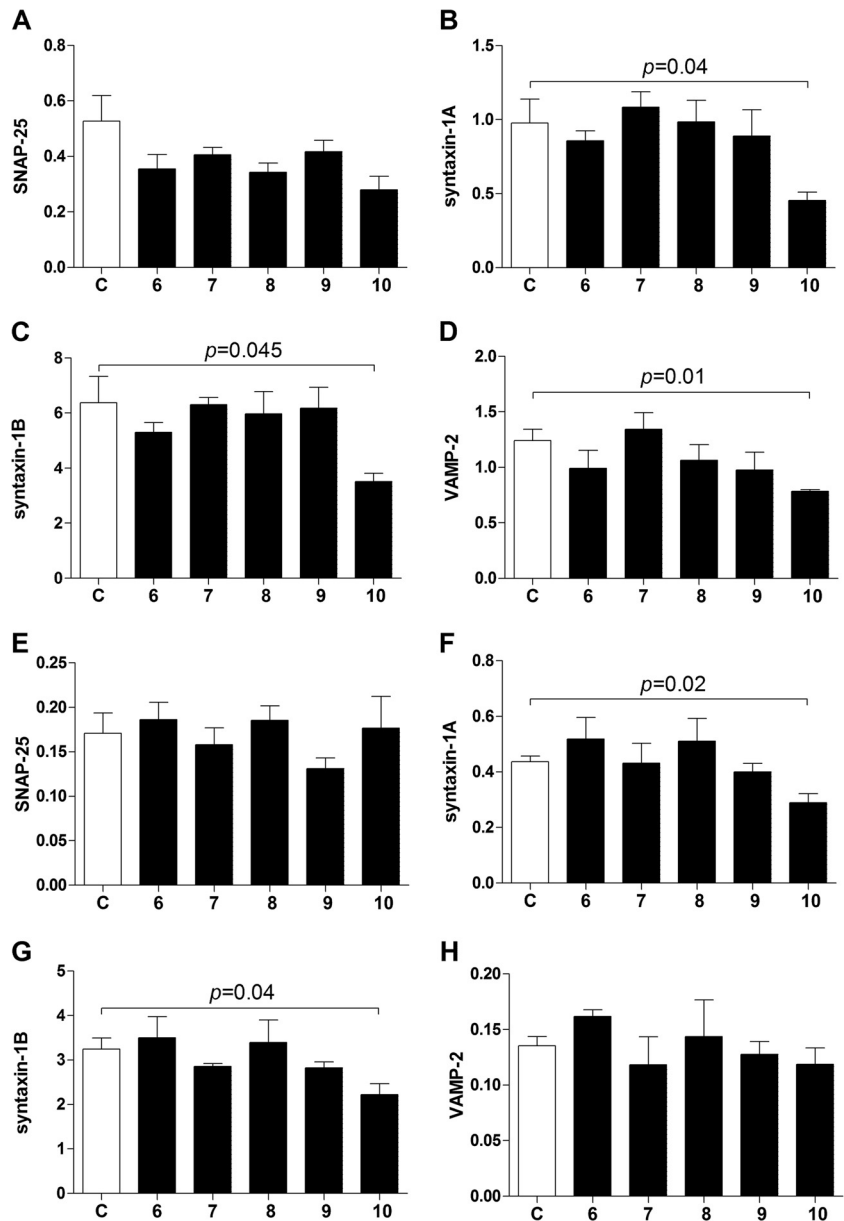


FIG. 3. Targeted mass spectrometry analyses of the SNARE complex in prion-infected mice. Individual values for the SRM-MS measured ratios (endogenous peptide/labeled peptide standard) of immunoprecipitated (SMI81 antibody) SNAP-25, syntaxin-1A, syntaxin-1B, and VAMP-2 in biochemically fractionated membrane-bound (A–D) and membrane-raft-associated (E–H) fractions of prion-infected mice at various weeks post-infection. Three mice are represented at each time point. All data show mean \pm S.E. (Student’s unpaired *t* test).

SNAP-25 ELISA-measured levels (membrane-bound fractions: $\rho = 0.865$, $p < 0.001$ for SP12 coating and $\rho = 0.839$, $p < 0.001$ for SMI81 coating), indicating that the two methods are comparably reproducible and sensitive. For membrane-raft-associated fractions, there was a moderate correlation between the IP-MS- and ELISA-measured levels of SNAP-25 ($\rho = 0.389$, $p < 0.05$ for SP12 coating and $\rho = 0.478$, $p = 0.008$ for SMI81 coating).

A major advantage of the IP-MS method is that because the SNARE complex proteins are highly conserved, the method can be applied directly, without modification of brain tissue samples from other species. To demonstrate this, we measured levels of SNARE proteins in mice with prion disease in which synaptic degeneration is known to occur. We used tg37 mice in which the time course of synapse loss and clinico-

pathological course are well mapped, with onset at around 7 weeks post-inoculation or 9 w.p.i., progressing to neuronal loss at 10 w.p.i. and death from prion disease at 12 w.p.i. when inoculated with Rocky Mountain Laboratory prions or normal brain homogenate at ~ 4 weeks of age as described in Refs. 20 and 21. We sampled brains from prion-infected tg37 mice and controls 6, 7, 8, 9, and 10 w.p.i. and fractionated these according to solubility. Levels of proteins immunoprecipitated with SMI81 were quantified with SRM-MS. Similar to results from human brain tissue, all SNARE complex proteins were observed in the membrane-bound and membrane-raft-associated protein fractions. SNAP-25 levels did not show any significant change with time after prion infection in any of the protein fractions. However, levels of the SNAP-25 interacting proteins syntaxin-1A and syntaxin-1B were signifi-

cantly decreased 10 weeks after prion infection (Figs. 3B, 3C, 3F, and 3G; supplemental Table S6) relative to uninfected controls. For VAMP-2, the measured levels were significantly decreased 10 weeks after prion infection in the membrane-bound protein fractions, but not in the membrane-raft-associated fractions (Figs. 3D and 3H, supplemental Table S6).

DISCUSSION

In this paper we describe an approach for the concurrent measurement of levels of all SNARE complex proteins from individual human and mouse brain tissue samples. The main advantage of the IP-MS method relative to immunocytochemistry, Western blotting, and ELISA methods that have been used to study pathological changes in SNARE complex protein expression is its versatility. With the IP-MS method, all SNARE proteins, including modified forms, can be quantified simultaneously, which is an advantage over existing methods. The protein target can be altered or expanded by changing antibodies, adding stable-isotope-labeled standards of modified protein forms or protein complex interaction partners, or changing the fractionation method to study different subtypes of cells or specific cell compartments (13). This is of particular interest when studying the SNARE complex proteins, as it has been shown that different combinations of SNARE protein isoforms interact with dissimilar affinities and are differentially distributed in defined areas of the central nervous system, suggesting specialized functions of these isoforms (29). Other SNARE protein isoforms such as SNAP-25A and -25B have very different expression during embryonic and early postnatal development: SNAP-25A is the predominant species during embryonic and early postnatal development, whereas SNAP-25B transcripts increase dramatically after the first postnatal week to become the majority of SNAP-25 mRNA in adult brain (38, 39). Other studies have shown that different SNARE protein isoforms could have specialized roles in the neurosecretory process (40).

Recently, Varjosalo *et al.* demonstrated the technical feasibility of obtaining high-quality maps of human protein interactomes using immunopurification combined with mass spectrometry (41). In their study they showed that very high within-laboratory and interlaboratory IP-MS reproducibility can be achieved. Our study focused on four SNARE proteins, including two isoforms of syntaxin-1 (A and B), but there are several very interesting interaction partners that could be added to the method without major changes. Interestingly, our results with mice corroborate earlier data indicating SNARE complex destabilization in prion-infected mice (21). These data support the notion that the described IP-MS method can be utilized to investigate the stability of the SNARE complex and the strength of association between the different SNARE protein forms or other interaction partners. The study involving mouse brain tissue also confirmed another important aspect of the presented IP-MS method. The mechanism and structure of SNARE proteins are widely evo-

lutionarily conserved (42, 43). Thus, it is possible to apply the method that we have developed for human brain tissue directly to brain tissue samples from several other species often used as animal models (e.g. rat, mouse, dog, pig, and zebrafish) without major modifications.

In summary, we have demonstrated that it is possible to use IP-MS to measure levels of four SNARE complex proteins in parallel. The applicability of the IP-MS method was demonstrated in a comparison of SNARE complex protein levels in brain tissue from AD patients and age-matched controls, as well as in a study of SNARE complex protein levels in brain tissue from prion-infected mice. Furthermore, the high correlation between the IP-MS-measured levels of SNAP-25 and the SNAP-25 levels measured with sandwich ELISA indicates that the two methods are comparably reproducible and sensitive. The presented method could be used to study the synaptic pathology in neuropsychiatric or neurodegenerative diseases and evaluate drugs aimed at restoring synaptic function in various animal models, as well as in human postmortem tissue and possibly biological fluids.

Acknowledgments—We are grateful to Rita Persson and Dzemila Secic for their technical assistance. We are also grateful to Professor Lennart Brodin and Dr. Peter Löw for providing the polyclonal SNAP-25 antibodies.

* This work was supported by grants from the Swedish Brain Power Consortium, Swedish Alzheimer Foundation, Demensfonden, Eivind och Elsa K:son Sylvans stiftelse, Märtha och Gustaf Ågrens stiftelse, Gun och Bertil Stohnes Foundation, Stiftelsen Gamla Tjänarinnor and Svenska Läkaresällskapet, the Canadian Institutes of Health Research (MOP-14037 and CBG-101827), and Åhlén-stiftelsen.

§ This article contains supplemental material.

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