A Comprehensive Identification of Synaptic Vesicle Proteins in Rat Brains by cRPLC/MS-MS and 2DE/MALDI-TOF-MS

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Proteomic analyses of synaptic vesicle fraction from rat brain have been performed for the better understanding of vesicle regulation and signal transmission. Two different approaches were applied to identify proteins in synaptic vesicle fraction. First, the isolated synaptic vesicle proteins were treated with trypsin, and the resulting peptides were analyzed using a high-pressure capillary reversed phase liquid chromatography/tandem mass spectrometry (cRPLC/MS/MS). Alternatively, proteins were separated by two-dimensional gel electrophoresis (2DE) and identified by matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF/MS). Total 18 and 52 proteins were identified from cRPLC/MS-MS and 2DE-MALDI-TOF-MS analysis, respectively. Among them only 2 proteins were identified by both methods. Of the proteins identified, 70% were soluble proteins and 30% were membrane proteins. They were categorized by their functions in vesicle trafficking and biogenesis, energy metabolism, signal transduction, transport and unknown functions. Among them, 27 proteins were not previously reported as synaptic proteins. The cellular functions of unknown proteins were estimated from the analysis of domain structure, expression profile and predicted interaction partners.

Key Words : Synaptic membrane proteome, Micro liquid chromatography electrospray ionization tandem mass spectrometry, Two-dimensional gel electrophoresis, Matrix-assisted laser desorption mass spectrometry, Rat brains

Introduction

Neuronal transmission in the central nervous system occurs at synaptic junctions. Incoming electronic signals from pre-synaptic neurons induce the release of neurotransmitters in synaptic vesicles to synaptic junction, and then the released neurotransmitters bind to corresponding receptors to activate the postsynaptic neurons. Synaptic vesicles are the key organelles involved in synaptic functions such as uptake, storage and stimulus-dependent release of neurotransmitters.[1,2] The trafficking cycle of synaptic vesicles consists of the transport of neurotransmitters into synaptic vesicles, clustering and docking of the vesicle in front of the active zone, priming and fusion of the vesicle with the plasma membrane and recycling by endocytosis. Proteins involved in the biogenesis and transport process of synaptic vesicles have been identified from genetic as well as biochemical analyses. The synaptosome-associated protein receptor (SNARE) complex, consisting syntaxin,[3] synaptobrevin (VAMP), SNAP25,[4] and synaptotagamins,[5] is necessary for the docking and fusion of synaptic vesicles with the plasma membrane. Cytoskeleton proteins, such as myosin-V, rab 6 and KIF3A, are involved in the transport of vesicle through the cytoplasm.[6] Septins are involved in the targeting of vesicles to plasma membrane.[7,8] Proteins implicated in the attachment of vesicle to acceptor membranes are Rab,[9,10] rabkinesin,[11] rabadaptin,[12,13] rabphilin,[14-16] Rim,[17] EEA1[18,19] and Uso1p/p115.[20,21] The vATPase complex, which provides energy for sequestering neurotransmitters in synaptic vesicles, is also highly expressed in the synaptic vesicle.[22] In addition, transporters, receptors, and receptor-associated proteins had been identified in synapse.[23]

Recently, global analyses of proteins from whole organism, specific tissues or organelles have been performed due to the recent advance in high-throughput mass spectrometric techniques.[24-26] Proteomes of human and mouse brain tissues have been analyzed using 2DE and MALDI-TOF-MS, and 200-450 proteins were identified.[27-32] Among them, only a few proteins involved in the trafficking of synaptic vesicles or signal transduction were identified.[33] Analyses of proteins in subcellular fraction from neuronal tissue, such as synaptosome or synaptic vesicles, were also carried out to identify proteins directly associated with synaptic vesicles.[34-36] Also, synaptosomal proteins from squid optic lobe have been separated using 2DE, and highly expressed proteins were

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Abbreviations: 2DE, two-dimensional gel electrophoresis; **ESI**, electrospray ionization; **MALDI**, matrix-assisted laser desorption ionization; **TOF**, time-of-flight; **cRPLC**, capillary reversed phase liquid chromatography; **QIT**, quadrupole ion trap; **IEF**, isoelectric focusing

identified.[37] Majority of these proteins were cytosolic enzymes, cytoskeletal proteins, or molecular chaperones. However, only a small number of proteins involved in synaptic architecture and transmitter recycling were identified. In addition, 36 proteins involved in vesicle maintenance, recycling and energy metabolism were identified from 2DE and LC/MS/MS analysis of mouse brain synaptic vesicles.[38] Also 452 proteins were identified from of postsynaptic density fraction from rat brain by LC/MS/MS analysis.[39] When the proteins from these analyses were compared, uniquely identified proteins in each analysis were found, indicating that each analysis identified only a fraction of protein in synaptic vesicle.

In this study, we have analyzed proteins associated with synaptic vesicles of rat brain in order to identify novel proteins involved in the cellular function of synaptic vesicles and components of signal transduction. To address the limitation of each MS-based proteome analysis methods, we have used 2DE-MALDI-TOF as well as cRPLC/MS/MS for the analysis of synaptic vesicle proteome. From these analyses 68 proteins were identified and categorized by their functions, and the functions of newly identified proteins were estimated. These data would provide information regarding to the molecular machinery of synaptic vesicle trafficking.

Experimental Section

Materials. Immobilized pH gradient (IPG) strips, CHAPS, DTT were purchased from Amersham Biosciences (Uppsala, Sweden). Acrylamide and bis-acrylamide cocktail solution was obtained from Bio-Rad (Hercules, USA). Urea, thiourea, iodoacetamide and the other reagents for the polyacrylamide gel preparation were from Sigma (St. Louis, USA). Reagents of silver staining were purchased from Merck (Darmstadt, Germany). The Sprague-Dawley rats (250-300 g) were purchased from Samtako (Seoul, Korea). The animals were sacrificed by decapitation and the whole brain was isolated. The brain samples were stored at −80 °C until analyzed.

Isolation of synaptic vesicles. The synaptic vesicle fraction was isolated from rat brains as described by Huttner *et al.*[40] Briefly, whole brain tissue (0.5 g) was homogenized with 10 strokes at 2,100 rpm using a glass/Teflon homogenizer in 5 mL of buffer A (4 mM HEPES, 320 mM sucrose, pH 7.3). The membrane fraction from the brain tissue extract was further separated by ultracentrifugation in 50-800 mM sucrose gradient for 5 hr at 65,000 × g, and a synaptic vesicle fraction in the 200-400 mM sucrose region was collected. Proteins in the isolated synaptic vesicle fraction were precipitated in 20% TCA in acetone containing 0.2% DTT, and the precipitated proteins were dissolved with 40 mM Tris-HCl, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2 mM tributylphosphine, 1 mM EDTA containing protease inhibitor cocktail [1 tablet per 50 mL hydration solution (Roche Diagnostics, Penzberg, Germany)]. Protein concentration was determined by bicinchoninic acid method.[41]

Electron microscopy. Electron microscopic image of the isolated vesicle fraction was obtained by the same method as previously reported.[40] The isolated synaptic vesicle fraction was fixed in 120 mM sodium cacodylate buffer (pH 7.4) containing 2% glutaraldehyde. The fixed synaptic vesicles were recovered by centrifugation for 1 h at 234,000 × g, washed with 0.1 M veronal/acetate buffer (pH 7.4), and post fixed in 1% OsO_4 at 4 °C. Then, the samples were dehydrated in ethanol and propylene oxide. Silver sections were prepared on an Ultra-Cut 701701 microtome (Leica Microsystems, Nussloch, Germany), stained with uranyl acetate and lead citrate, and examined in a Philips 301 electron microscope (Philips Electron Optics, Eindhoven, Holland) operated at 80 kV.

Tryptic digestion of synaptic vesicle proteins. Synaptic vesicle fraction (30 μg of proteins) was dissolved in 300 μL of 100 mM ammonium bicarbonate buffer with 6 M urea and thermally denatured by incubation at 90 °C for 10 min. After the sample was cooled to room temperature, 0.6 μg of sequencing grade Lys-C (Roche Diagnostics, Indianapolis, USA) was added and incubated at 37 °C for 4 h. The Lys-C digested sample was diluted with 600 μL of 100 mM ammonium bicarbonate buffer, and further digested with trypsin (Promega, Madison, USA) with a substrate-to-enzyme ratio of 50:1 at 37 °C for 15 h. The resulting peptide mixture was applied onto a home-built micro-column (0.03" I.D. × 5 cm long, C_{18}-bonded particles, 5 μm particles, 300 Å pore size; Phenomenex, Torrance, USA), washed and desalted with 0.05% trifluoroacetic acid (TFA) and 0.2% acetic acid solution, and eluted with 90% acetonitrile and 0.1% TFA solution. The eluted peptide sample was completely dried and kept at −20 °C until required for cRPLC/MS/MS experiments.

cRPLC/MS/MS analysis. Tryptic peptides from synaptic vesicle fractions were separated using a high-pressure capillary RPLC system that had been described in detail elsewhere.[42] Briefly, mobile phases (solvent A: 0.05% TFA and 0.2% acetic acid in water; solvent B: 90% acetonitrile, 0.1% TFA in water) were delivered into a capillary column (75 μm ID × 360 μm OD × 1 m in length) by two ISCO syringe pumps (Lincoln, USA) at the operation pressure of 10,000 psi with an exponential gradient of 0-80% of solvent B. A quadrupole ion trap (QIT) mass spectrometer (LCQ advantage; ThermoFinnigan, San Jose, USA) equipped with a home-built nanoESI interface was used to analyze the peptides from the capillary RPLC system. To identify the eluted peptides, the QIT-MS was operated in data-dependent tandem MS mode where the four most abundant ions detected in a precursor MS scan were dynamically selected for subsequent MS/MS experiments, simultaneously incorporating dynamic exclusion option to prevent reacquisition of MS/MS spectra of the same peptides. The MS and MS/MS data from QIT-MS experiments were analyzed using the SEQUEST program (Thermofinnigan, San Jose, USA) against a database, which was constructed by combining the IPI rat database (ftp://ftp.ebi.ac.uk/pub/databases/IPI/current) and a common contaminant database (http://www.ncbi.nih.

gov). Database search was performed by using m/z tolerance of 3 Da for precursor ions, and 1 Da for fragment ions. The search results were subsequently evaluated by PeptideProphet[13] and peptides having probability score of higher than 0.5 were exported to a home-built software,[14] where the peptide IDs were further matched to proteins. Proteins having 3 or more different peptide sequences were considered as being correctly identified. Proteins identified by one or two peptides were further analyzed by manual inspection of the corresponding MS/MS spectra to minimize false positive identification.

Electrophoresis. 2DE was performed by the same methods as previously reported.[15] About 1 mg proteins of the solubilized synaptic vesicle fraction were mixed with 2% (v/v) isoelectric focusing gel buffer (pI 3-10), and then applied on an immobilized linear gradient strip (pI 3-10; Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing was performed at 150 V for 1 h, at 500 V for 30 min, at 1,000 V for 30 min, and then kept constant at 8000 V for 10 h at 20 °C. For the second dimension electrophoresis, the IPG strips were incubated in equilibration buffer (1.5 mM Tris-HCl, 6 M urea, 2% SDS, 30% glycerol, 2% DTT, pH 8.8) for 15 min, and then incubated for 15 min in the equilibration buffer containing 2.5% iodoacetamide. The equilibrated IPG strips were transferred to the second dimension SDS-PAGE of 10% gels. Electrophoresis was carried out at 10 °C using a Hoeffer TM SE 600 system (Amersham Biosciences, Uppsala, Sweden). The proteins in 2DE gels were visualized by silver staining method as described previously.[16] One of the five gels was chosen as the master gel, and used for the automatic matching of spots in other 2DE gels.

MALDI-TOF/MS and database search. The gel slices containing the stained protein spot were washed three times with 25 mM ammonium bicarbonate and 50% acetonitrile solution for 1 h, dried, and proteins in gels were digested with 12.5 μg/mL sequencing grade trypsin (Promega, Madison, USA) in 25 mM ammonium bicarbonate for overnight at 37 °C. The peptides from the gel were extracted with 60% acetonitrile solution containing 0.1% trifluoroacetic acid. After evaporation of the solvent, peptides were dissolved in 0.05% trifluoroacetic acid, 5% acetonitrile solution. One μL of the peptide mixture was mixed with the same volume of the matrix solution (5 mg/mL α-cyano-4-hydroxy-cinnamic acid solution in 50% acetonitrile containing 0.1% trifluoroacetic acid), and the mass values of peptides were determined by MALDI-TOF/MS (Voyager DE-PRO, Applied Biosystems, Foster City, U.S.A.) mass spectrometer. The peptide masses were compared with the theoretical peptide masses of all available proteins from *Rattus norvegicus* and *Mus musculus*. Monoisotopic masses were used with a mass tolerance of 50 ppm.

Database analysis of unknown function proteins. The function of proteins, which were not listed in gene ontology database (http://www.ebi.ac.uk/ego/), was further analyzed using PSORTII (http://psort.nibb.ac.jp/ form2.html) database for the cellular localization, PROSITE (http://www.expasy.org/prosite) and CDD (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=cdd) databases for the identification of structural domain, STRING database (http://string.embl.de/) for the identification of interacting proteins. Also, potential orthologs of the unknown proteins were analyzed by the prediction of protein-protein interactions using NANO_PID database (Nanomics, Inc.). Since the protein-protein interaction databases including NANO_PID did not reach saturation level, the prediction of potential orthologs based on the best match resulted in substantial limitations.[17] Indeed, orthology can be one-to-many or many-to-many relationship.[18] In this study, all matches with E-values lower than 10^{-10} were considered as potential orthologs.

Results

Purification of synaptic vesicle and proteome analysis. The synaptic vesicles were purified from the homogenate of rat brain tissue by differential centrifugation and ultracentrifugation in sucrose gradient. About 1.2 mg of synaptic vesicle proteins was obtained from 13.2 g of brain tissue, and the yield of synaptic vesicle was comparable to the reported value.[10] The electron microphotograph of the purified synaptic vesicles shows a near homogeneous population of vesicle structure within the range of 40-70 nm diameter (Fig. 1A, × 16,500; Fig. 1B, × 65,000), which is consistent with the reported size of synaptic vesicle.[19] Subcellular

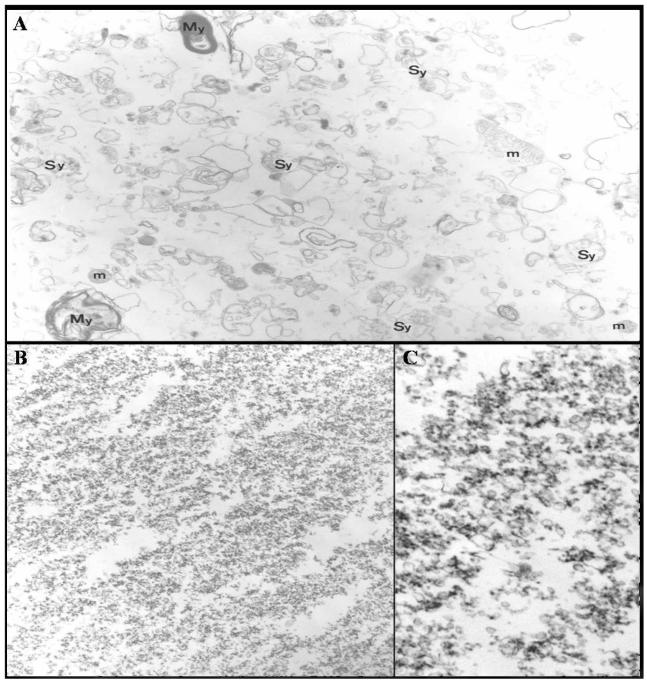

Figure 1. The electron micrograph of synaptic vesicles. Synaptic vesicles from the 200-400 mM sucrose region of gradient fractions were collected at 65,000 × g and the membrane pellet was analyzed. The image, shown at 60,000 ×, displays a homogenous population of intact synaptic vesicles. Subcellular particles from the crude synaptosomal pellet, × 16,500 (A). Highly purified synaptic vesicle fraction (SGV), × 16,500 (B) and × 65,000 (C). *Sy*, synaptosomes; *My*, myelin sheath; *m*, mitochondria

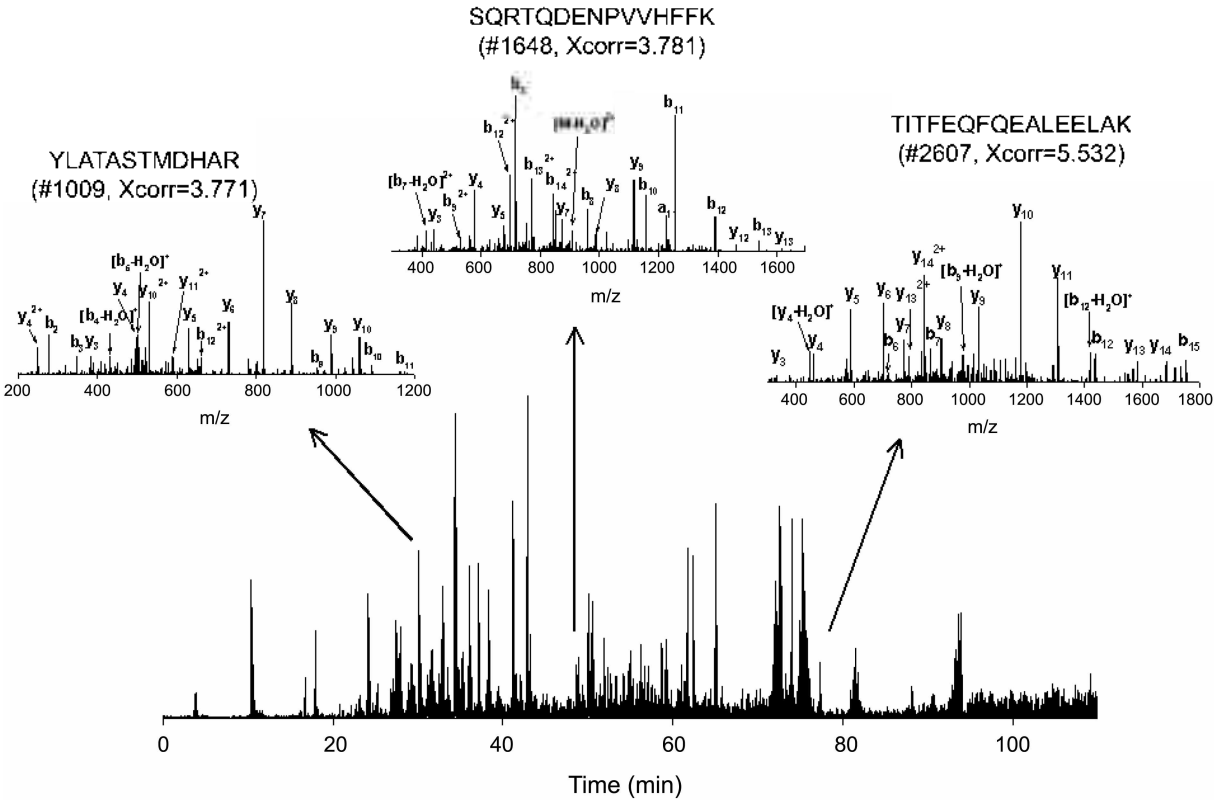

Figure 2. Chromatogram of tryptic digestion of synaptic vesicles in cRPLC. The peak height in the chromatogram represents relative abundance. Confident peptide assignments were exemplified by a few representative MS/MS spectra.

organelles with higher density such as mitochondria or nucleus were removed in high speed centrifugation step (Fig. 1C), and not observed in the electron microscopy of the purified sample.

Proteins from synaptic vesicle fraction were precipitated and treated with trypsin, and the resulting peptides were analyzed by cRPLCMS/MS method. A chromatogram of an LC/MS/MS experiment using tryptic peptides from the synaptic vesicle proteins was shown in Figure 2 with three representative MS/MS spectra. As a whole, seventy-five proteins were identified from the initial database search using the filtering criteria described in "Experimental Section", and 18 proteins were finally identified as positive hits by manually inspecting the MS/MS spectra after removing low confidence proteins. For the proteome analysis using 2DE, proteins from the prepared synaptic vesicle fraction was extracted from membrane and fully dissolved before further analysis. Optimum separation of synaptic vesicle proteins in 2DE was obtained by the membrane extraction with high concentration of urea, thiourea, DTT and stepwise application of high voltage during IEF as described in "Experimental Section" (Fig. 3). Using the optimized electrophoresis condition, 500 detectable protein spots were separated in 2DE gel (Fig. 4). Among them, 150 spots, which were well separated with measurable intensity in silver stained gel, were picked and undergone mass analysis.

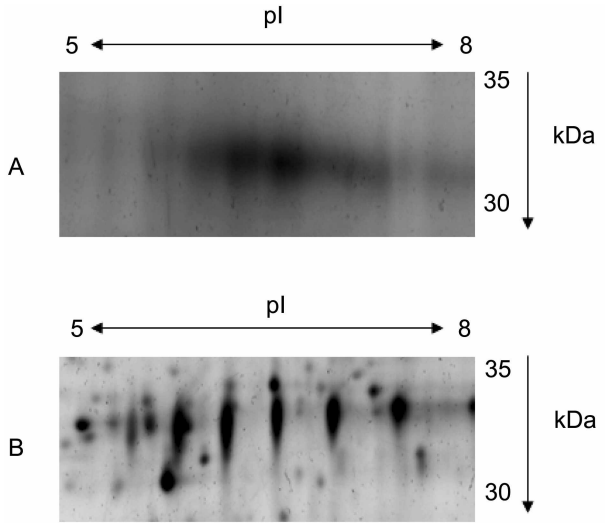

Figure 3. Improvement of resolution in 2DE gel. (A) 2DE image of synaptic vesicle proteins prepared from general lysis solution (8 M urea, 4% CHAPS, 20 mM DTT). (B) 2DE image of synaptic vesicle proteins prepared from lysis solution (7 M urea 2 M Thiourea, 4% CHAPS, 2 mM tributylphosphine) and de-contamination.

Identification of protein by 2DE/MALDI-TOF-MS and cRPLC/MS/MS analysis. The protein spots isolated from the 2DE gel were digested by trypsin and the resulting peptides were analyzed by MALDI-TOF/MS. Among the 150 protein spots detected on 2DE gel, 52 spots were successfully identified. Others were poorly recovered from the gel and generated only a few MS peaks which were not

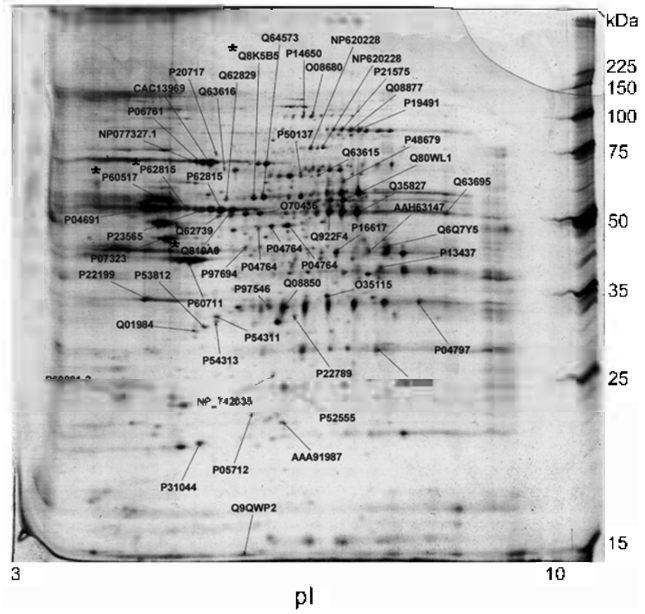

Figure 4. 2D map of rat brain synaptic vesicle protein. Proteins from rat synaptic vesicles brains were separated on a pH 3-10 nonlinear IPG strip, followed by a 10% PAGE, as described in Materials and Methods. The gels were stained with silver nitrate, and the visualized spots were analyzed by MALDI-MS. The accession numbers from protein database (http://kr.expasy.org/sprot/ or http://www.ncbi.nlm.nih.gov/) indicate the identified protein spots.

sufficient for mass fingerprinting with statistical confidence. The accession numbers of 52 identified protein spots are indicated on 2DE gel (Fig. 4), and the identified proteins are listed in Table 1 along with name, function, and the accession number, isoelectric point, molecular weight, percent of coverage, Mowse score, and the sequences of peptide hits. Proteins that were identified by cRPLC/MS/MS database analysis are also indicated in Table 1. The identified proteins have more than 95% confidence level. Only two proteins (Vacuolar ATP synthase subunit B and actin) were identified by both methods. The low percentage of overlapping proteins in the two analytical methods indicates that certain proteins are preferentially well-resolved in particular analytical methods, and different analytical methods should be applied to identify the maximum number of proteins in specific sample.

Among the identified proteins, proteins related to the vesicle trafficking were of particular interest. The SNARE complex has been responsible for the fusion of synaptic vesicles to plasma membrane, and syntaxin, SNAP-25, synapsin, and VAMP-2 are known as the key components of SNARE complex.[50] All of these components involved in the trafficking and fusion of synaptic vesicles are identified in this study. Besides, proteins implicated to vesicle trafficking such as dynamin and r-vsp33 are detected. Among the vesicle related proteins, Vacuolar ATP synthase subunit B, which is responsible for the energy production and acidifying intracellular compartments, showed strong intensity of protein spot in 2DE gel indicating that the protein is highly expressed in synaptic vesicles (data not shown).

The major excitatory and inhibitory neurotransmitters in the brain, glutamate and GABA, activate both ionotropic (ligand-gated ion channels) and metabotropic (G protein-coupled) receptors, and are generally associated with neuronal communication in the mature brain.[51] In this study, 12 kinds of receptors, transporters and signaling proteins are identified. They are mineralocorticoid receptor, which is one of corticosteroid receptors and highly expressed in the hippocampus,[52] ephrin type-A receptor, G-protein α_{13}, β_1, β_2, and GABA receptor-associated protein and glutamate transporter. Specially GABA(A) receptor-associated protein and glutamate transporter (GLT1b) showed high expression level in 2DE gel analysis. Also, senescence marker protein-30 (SMP-30) and calmodulin 1, which are related to calcium signaling, are identified.

Among the identified proteins, cytoskeleton proteins, such as actin, and α-internexin are highly expressed proteins and frequently observed in proteomic analysis.[53] They are also related to the regulation of morphology and synaptic function. Especially, actin and its binding partners are involved in the release of neurotransmitters at synapses.[54] Besides cytoskeleton proteins, various enzymes involved in metabolism of carbohydrate, nucleic acid or lipid, energy generation or protein processing are identified. It is noticeable that Hect domain/RLD4 (Q810A0), which is involved in ubiquitin cycle, is highly expressed based on the protein intensity in 2DE gel (data not shown). Since protein ubiquitination is involved in the regulation of vesicle trafficking as well as in the coordination of protein turnover with synaptic function in nerve terminals,[55] Hect domain/RLD4 may be responsible for the biogenesis of synaptic vesicles in brain as previously suggested.

Discussion

Comparison of identified proteins with previously reported synaptic proteins. Previous analyses of rat synaptic proteome reported 36 to 1131 proteins depending on the sample and analysis methods. When the proteins identified in this study are compared with them, less than 10% of the reported proteins from synaptosme or post synaptic density fraction are overlapped with the proteins identified in this study (Table 2). The low percentage of overlapped proteins in different reports suggests that the methods for sample preparation and mass analysis significantly affect the kinds and range of identified proteins in each proteome analysis. It is noticeable that 25% of proteins from membrane enriched fraction of synaptic vesicle[38] are identified in this study (Table 3). The relatively high percentage of overlap might be due to the fact that both analyses used the same membrane enriched fractions. Twenty seven proteins out of 68 identified proteins have not been reported previously. They include proteins in involved in vesicle trafficking (Q008850), signaling process (P22199, Q6Q7Y5, O08680, Q03336, Q80WL1, XP_001073968), or structural protein (XP_341747). Particularly, 6 out of 9 unconfirmed

Table 1. Tabular presentation of proteins found in SGV fraction

ID[a]	Name	Function	pI	MS	%C	Mowse	Peptides
Vesicle, synapse and neuron							
Q08850	Syntaxin-4	Docking of synaptic vesicles	5.8	34209	28	8.95E+02	-
P60881-2	SNAP-25A	Molecular regulation of neurotransmitter release	4.7	23315	45	2.75E−03	-
P21575	Dynamin-1	Vesicular trafficking processes	6.3	95928	14	9.89E+03	-
Q08877	Dynamin-3	Vesicular trafficking processes	7.6	95596	4	7.13E+02	-
Q63615	Vacuolar protein sorting 33A	Protein trafficking, membrane docking/fusion	6.6	67514	12	1.93E−03	-
Q63616	Vacuolar protein sorting 33AB	Protein trafficking, membrane docking/fusion	6.4	70694	18	1.14E−05	-
P19491	Glutamate receptor 2 [Precursor]	Communication from a neuron to a target	7.1	98689	13	1.56E−05	-
P63045	VAMP-2	Ttargeting and/or fusion of transport vesicles to their target membrane	8.0	12690	16.3	-	PPAAPAGEGGPPAPPPNLTSNR
P09951-2	Synapsin-1B	Coating of synaptic vesicles. Releasing of neurotrasmitter	9.8	69909	7.8	-	DASPGRGSHSQTPSPGALPLGR; T]YATAEPFIDAKYDVR: TYATAEPFIDAKYDVR : V]LLVIDEPHTDWAK;
P02688-1	Isoform 1 of Myelin basic protein S	Assembly of an optimized, biochemically functional myelin membrane	11.2	21500	66.2	-	D]ENPVVHFFKNIVTPR; DENPVVHFFKNIVTPR; DSHTRTTHYGSLPQK; F]FSGDRGAPK; F]SWGAEGQKPGFGYGGR; FFSGDRGAPK; FSWGAEGQKPGFGYGGR; G]RGLSLSR; G]SKYLATASTMDHAR; GAYDAQGTLSK; GRGLSLSR; H]GSKYLATASTMDHAR; H]RDTGILDSIGR: HGSKYLATASTMDHAR; HRDTGILDSIGR: HRDTGILDSIGRF; HYGSLPQK; L]ATASTMDHAR: LATASTMDHAR; N]IVTPRTPPPSQGK; NIVTPRTPPPSQGK; P]VVHFFKNIVTPR; Q]SRSPLPSHAR; QSRSPLPSHAR; S]QRTQDENPVVHFFK; S]QRTQDENPVVHFFKNIVTPR; SQRTQDENPVVHFFK; SQRTQDENPVVHFFKNIVTPR; T]QDENPVVHFFK; T]QDENPVVHFFKNIVTPR: T]THYGSLPQK; T]THYGSLPQKSQR; TQDENPVVHFFK: TQDENPVVHFFKNIVTPR: TTHYGSLPQK: TTHYGSLPQKSQR: TTHYGSLPQKSQRTQDENPVVHFFK; Y]LATASTMDHAR: YLATASTMDHAR;
P47709	Rabphilin-3A	Releasing of neurotransmitter	8.5	75832	4.7	-	ANSVQASRPAPASMPSPAPPQPVQPGPPGGSR
Receptors, transporters and signaling proteins							
P22199	Mineralocorticoid receptor	Receptor for mineralocorticoids and glucocorticoids	7.5	106738	12	8.62E−03	
P54311	$G\beta_1$	Modulator or transducer in various signaling systems	5.5	37393	40	1.54E+06	
P54313	$G\beta_2$	Modulator or transducer in various signaling systems	5.6	37331	47	4.72E+07	
Q6Q7Y5	$G\alpha_{13}$	Modulator or transducer in various signaling systems	8.4	44012	10	1.58E+02	
P31044	PEBP-1	Increases the production of choline acetyltransferase	5.5	20802	74	9.17E−04	
O08680	Ephrin type-A receptor 3 [Precursor]	Development of the central nervous	6.5	110229	9	4.44E−03	
Q8K5B5	Glutamate transporter GLT1b	Dicarboxylic acid transport	6.2	60918	24	5.49E−03	
P60517	GABA(A) receptor-associated	Transport of GABA(A) receptors	4.9	50164	25	1.79E−06	
Q03336	Senescence marker protein-30	Regulation of calcium signaling	6.2	12885	36	1.18E−02	
Q62772	Tenascin -X	Receptor binding and signal transduction	6.4	23561	31	1.22E−03	-

Table 1. Continued

ID[a]	Name	Function	pI	MS	%C	Mowse	Peptides
Q80WL1	Gliomedin	Transport of phosphate	7.7	59331	8	2.11E03	-
XP_001073968	Similar to calmodulin 1	control of a large number of proteins by Ca^{2-}	4.1	16814	45	-	A]DQLTEEQIAEFKEAFSLFDKDGDGTITTK; SJLGQNPTEAELQDMINEVDADGNGTIDFPEFLT MMAR; SLGQNPTEAELQDMINEVDADGNGTIDFPEFLT MMAR
Cytoskeleton and cell movement related protein							
P60711	Actin, cytoplasmic 1	In various types of cell motility	5.3	41751	48/5.6	2.09E−08	AGFAGDDAPRAVFPSIVGRPR
P23565	α-internexin	Morphogenesis of neurons	5.2	56116	17	6.06E+03	-
XP_341747	similar to tubulin polymerization-promoting	Promotes in vitro the polymerization of tubulinsy	9.4	23545	7.3	-	TJITFEQFQEALEELAK; TITFEQFQEALEELAK
Enzyme							
NP_742035	Glutathione s-transferase Mu5	Conjugating GSH with a electrophilic substrates	6.3	26629	27	4.81E+02	
Q64573	Liver carboxylesterase 4 [Precursor]	Detoxification of xenobiotics. activation of ester, amides	6.3	62275	17	3.65E+04	
P14650	Thyroid peroxidase [Precursor]	Oxidative stress	6.6	101461	14	6.94E+04	
P50137	Transketolase	Link glycolytic and pentose-phosphate pathways	7.2	67644	7	1.36E+02	
Q01984	Histamine N-methyltransferase	Degrading histamine	5.0	33942	18	4.04E+02	
Q6P7So	Pkm2	Glycolysis	8.0	52936	5.7	-	AATESFASDPILYRPVAVALDTKGPEIR
P09606	Glutamine synthetase 1	ATP + L-glutamate + NH_3 = ADP + phosphate − L-glutamine	6.6	42268	2	-	SASIRIPR
NP_955417	Dihydrolipoyl dehydrogenase	Component of the glycine cleavage system (By similarity).	7.9	54038	4.7	-	SDQPIDADVTVIGSGPGGYVAAIK
Energy generation and provide							
P25809	Creatine kinase, ubiquitous [precursor]	Transfer of phosphate between ATP and various phosphogens	8.7	47029	8.1	-	LSEMTEAEQQQLIDDHFLFDKPVSPLLTAAGMA R
P10719	ATP synthase subunit β, mitochondrial [precursor]	Producing of ATP from ADP in the presence of a proton gradient	5.2	56350	2.8	-	LJVLEVAQHLGESTVR ; LVLEVAQHLGESTVR
Q811A7	Lysosomal ATPase	Energy production	6.8	12034	13.4	-	A]SQSQGIQQLLQAEK
P62815	Vacuolar ATP synthase subunit B, brain isoform	Non-catalytic subunit of the peripheral V1 complex of vacuolar ATPase	5.6	56546	40/2.9	4.63E+10	YAEIVHLTLPDGTKR
P01946	Hemoglobin subunit α-1/2	Oxygen transport	7.9	15327	62	-	A]ADHVEDLPGALSTLSDLHAHK: A]ADHVEDLPGALSTLSDLHAHKLR: AADHVEDLPGALSTLSDLHAHK: FLASVSTVLTSKY; I]GGHGGEYGEEALQR: IGGHGGEYGEEALQR; LPGALSTLSDLHAHK; M]FAAFPTTKTYFSHIDVSPGSAQVK: MFAAFPTTKTYFSHIDVSPGSAQVK: P]TTKTYFSHIDVSPGSAQVK: T]YFSHIDVSPGSAQVK; TYFSHIDVSPGSAQVK: V]LSADDKTNIK; VLSADDKTNIK
P02091	Hemoglobin subunit β-1	Oxygen transport	8.0	15978	50		A]AVNGLWGKVNPDDVGGEALGR: AAVNGLWGKVNPDDVGGEALGR: LLVVYPWTQRYFDSFGDLSSASAIMGNPK: V]HLTDAEK; V]HLTDAEKAAVNGLWGKVNPDDVGGEALGR; V]VAGVASALAHKYH: VHLTDAEK; VHLTDAEKAAVNGLWGKVNPDDVGGEALGR; VVAGVASALAHKYH: Y]FDSFGDLSSASAIMGNPK: YFDSFGDLSSASAIMGNPK

Table 1. Continued

ID[a]	Name	Function	pI	MS	%C	Mowse	Peptides
Carbohydrate. Nucleic acid Metabolism							
P04764	α enolase	Glycolysis	6.2	47116	29	1.47E+09	-
P07323	γenolase	Glycolysis	5.0	47141	23	1.80E+04	-
P16617	Phosphoglycerate kinase	Glycolysis	7.5	44555	17	4.94E+02	-
P04797	GAPDH	Glycolysis	8.4	35836	27	4.85E+02	-
Lipid metabolism							
P13437	β-ketothiolase	Fatty acid beta-oxidation cycle	8.1	41871	19	2.43E+03	-
P22789	Alcohol sulfo-transferase A	Catalyzes sulfation of hydroxysteroids and xenobiotics	7.6	33252	22	8.28E+03	-
Nucleotide processing							
Q63520	Synaptonemal complex protein 3	Play important roles in chromosome condensation	8.7	29347	18	9.82E+02	-
Q63695	Octamer-binding TF [Fragment]	Transcription factor for snRNA etc	8.6	51223	8	1.33E+02	-
P48679	Lamin A	Components of the nuclear lamina	6.2	71642	19	2.44E+03	-
O70436	Mothers against DPP homolog 2	Regulation of transcription, DNA-dependent	6.1	52240	9	2.45E+02	-
Protein processing, folding and modification							
P20717	Protein-arginine deiminase II	Deimination of arginine residues of proteins	5.3	75357	15	1.13E+04	-
Q62829	Ser/Thr-protein kinase PAK 3	Activated kinase acts on a variety of targets	5.4	60711	3	1.04E+03	-
P53812	PtdIns transfer protein beta isoform	Transfer of PtdIns and phosphatidylcholine	5.5	31466	12	2.14E+02	-
P63018	Heat shock cognate 71 kDa	Chaperone	5.4	70872	10	1.69E+04	-
P06761	78 kDa GRP [Precursor]	Assembly of multimeric protein complexes	5.1	72348	23	2.69E+05	-
P52555	ERp29 [Precursor]	Processing of secretory proteins within the ER	6.2	28575	15	7.45E+02	-
P05712	Ras-related protein Rab-2A	Protein transport from the ER to the Golgi complex	6.0	22508	18	2.35E+02	-
P97694	Cytohesin 1	Promotes guanine-nucleotide exchange on ARF6	5.5	46274	20	2.18E+05	-
Q810A0	Hect domain and RLD 4	Involved in ubiquitin cycle	5.6	58813	12	3.25E+02	-
Unconfirmed function							
Q62739	RAB3A-interacting protein	Interacts with the N-terminal region of SSX2 (By similarity)	5.5	50951	11	1.83E+02	-
O35827	Fe65-like protein 2	Modulation of the internalization of beta-amyloid precursor[c]	7.5	54908	9	7.05E+02	-
Q9JIL9	Nibrin	Cardiomyocyte proliferation, DNA repair[c]	6.5	83305	17	1.13E+05	-
Q712J2	Bicaudal D [Fragment]	Dynein-dynactin interactions[c]	5.5	73562	12	9.40E+02	-
O35115	Four and a half LIM domains protein 2	Unknown, contains 4 LIM zinc-binding domains	7.3	32087	20	4.24E+02	-
P97546	Neuroplastin [Precursor]	Unknown, component of synaptic membrane	6.2	31293	25	1.78E+02	-
AAH63147	Unknown protein MGC:72671	Unknown, contains 3KH domains, nucleic acid binding	8.5	41293	28	1.87E+03	-
Q9QZA6	CD151 antigen	Assembly of the glomerular and tubular basement membranes [By similarity]	8.1	28353	5.1	-	Y]LATAYILVVAGV
XP_221941	similar to zinc finger protein 469	Unknown, contain BAH domains	8.6	220180	1.6	-	A]SEVSSHSYNTDSDEDEDLLKNSWSAQGPSSS

[a]Protein identification number from expasy (http://kr.expasy.org.srs5/) or NCBI (http://www.ncbi.nlm.nih.gov/). **pI**, isoelectric point; **MS**, molecular weight of protein (Dalton); **%C**, percent sequence coverage by measured masses; **Mowse**, score from MS-FIT database (http://prospector.ucsf.edu/)

Table 2. Comparison of proteomic analysis of synaptic vesicle fraction

	Stevens, 2003[36]	Li, 2004[67]	Jordan, 2004[39]	Coughenour, 2004[38]	Witzmann, 2005[68]	Schrimpf, 2005[69]	Li, 2005[70]	This study
Sample	Synaptic plasma membrane	PSD	PSD	Synaptic vesicle membrane	Synaptosome	Synaptosome	PSD	Synaptic vesicle
Method	1D, MALDI MS & LC MS/MS	2D, MALDI MS/MS & ICAT, LC MS	LC MS/MS	2D, LC MS/MS	2D, MALDI MS & LC MS/MS	ICAT LC MS/MS	ICAT LC MS/MS	2D, MALDI MS & LC MS/MS
ID	108	170	452	36	317	1131	130	68
Uniq.	Q08850, P22199, Q6Q7Y5, O08680, Q03336, Q80WL1, Q64573, P14650, P22789, Q63520, Q63695, O70436, P20717, P53812, Q810A0, P97694, O35827, O35827, O35115, Q62739, Q9QZA6, XP_341747, AAH63147, XP_221941, XP_001073968, NP_955417, Q811A7							

ID, Total number of identifies protein; **Uniq**., Unique protein list; **PSD**, Postsynaptic density

Table 3. List of membrane protein

Name	Domains[a] (predicted)	Subcellular location[b]	TM	PTM	M
Syntaxin-4	SynN, t-SNARE,	Membrane; single-pass type IV membrane protein (Potential).	1	-	I
SNAP-25A	CC, t-SNARE	Synaptic vesicle	-	Palmitoylated	P
Vacuolar protein sorting 33A	Sec1	peripheral membrane protein associated with late endosomes/lysosomes			P
Vacuolar protein sorting 33B	Sec1	peripheral membrane protein associated with late endosomes/lysosomes			P
Glutamate receptor 2 [Precursor]	Signal peptide, ANF_receptor, PBPe, LIg_chan	membrane; multi-pass membrane protein	3	Palmitoylated	I
VAMP-2	Coiled coil, Synaptobrevin,	Type IV membrane protein. Neuronal synaptic vesicles	1		I
Ephrin type-A receptor 3 [Precursor]	Signal peptide, EPH_lbd, FN3, TyrKc, SAM	type i membrane protein. the short isoform is secreted	2		I
Glutamate transporter GLT1b	SDF	membrane; multi-pass membrane protein	10		I
GABA(A) receptor-associated	MAP1_LC3	membrane-associated through a lipid anchor	-	Lipid modification	P
Gliomedin	Collagen, OLF	membrane; single-pass membrane protein	1	Hydroxylated, disulfide bonding	I
Liver carboxylesterase 4 [Precursor]	COesterase	Microsomal membrane, lumen of endoplasmic reticulum	1		I
Thyroid peroxidase [Precursor]	Signal peptide, An_peroxidase, CCP, EGF_CA,	single-pass type i membrane protein	1	Heme	I
ATP synthase βchain, [Precursor]	ATP-synt_ab_N, AAA, ATP-synt_ab_C	mitochondrion	-		P
α enolase	Enolase_N, Enolase_C	can translocate to the plasma membrane	1		I
γ endase	Enolase_N, Enolase_C	can translocate to the plasma membrane	1		I
Dihydrolipoamide dehydrogenase	GIDA, Pyr_redox	mitochondrion	1		I
ERp29 [Precursor]	Signal peptide, Erp29	endoplasmic reticulum lumen	1		I
Bicaudal D [Fragment]	Coiled coil	associated with intracellular membranes	-		P
CD151 antigen	Tetraspannin	multi-pass membrane protein	3		I

[a]Domaines were determined using SMART (http://smart.embl-heidelberg.de/) and Pfam (http://www.sanger.ac.uk/Software/Pfam/). Domain abbreviation key: **SynN**, Syntaxin N-terminal domain; **t-SNARE**, target membrane soluble N-ethylmaleimide attachment protein receptor; **CC**, Coiled coil; **Sec1**, Domain named after the *S. cerevisiae* SEC1 gene product: **ANF_receptor**, Atrial natriuretic factor receptor; **PBPe**, Eukaryotic homologues of bacterial periplasmic substrate binding proteins; **LIg_chan**, Ligand-gated ion channel; **EPH_lbd**, Ephrin receptor ligand binding domain: **FN3**, Fibronectin type III domain; **TyrKc**, Tyrosine kinase, catalytic domain; **SAM**, Sterile alpha motif; **SDF**, Sodium:dicarboxylate symporter family; **MAP1_LC3**, Microtubule associated protein 1A/1B, light chain 3; **Collagen**, Collagen triple helix repeat; **OLF**, Olfactomedin-like domain; **COesterase**, Carboxylesterase; **An_peroxidase**, Animal haem peroxidase ; **CCP**, Domain abundant in complement control proteins; **EGF_CA**, Calcium-binding EGF-like domain; **ATP-synt_ab**, ATP synthase alpha/beta family; ATP-gua_PtransN, ATP:guanido phosphotransferase, N-terminal domain; **ATP-gua_Ptrans**, ATP:guanido phosphotransferase, C-terminal catalytic domain; **ATP-synt_ab_N**, ATP synthase alpha/beta family, beta-barrel domainAAA; **ATP-synt_ab_C**, ATP synthase alpha/beta chain, C terminal domain; **Enolase_N**, Enolase, N-terminal domain; **Enolase_C**, Enolase, C-terminal domain; **GIDA**, Glucose inhibited division protein A; **Pyr_redox**, Pyridine nucleotide-disulphide oxidoreductase; **Erp29**, Endoplasmic reticulum protein ERp29, C-terminal domain; **Tetraspannin**, Tetraspanin family. [b]Subcellular location were determined using SOURCE (http://source.stanford.edu).

proteins are newly identified proteins in this study. Analysis of the molecular function of these proteins would provide detailed information on the process of vesicle trafficking in synapses.

Functional estimation of unknown proteins. Among the 68 identified proteins from rat synaptic vesicle fraction, the cellular function of 9 proteins not confirmed (Table 1). The function of these proteins was further estimated from the putative binding partners of these proteins, expression profile or structural homologues. Among them, 3 proteins (Bicaudal D, Neuroplastin and RAB3A-interacting protein) are implicated to be involved in vesicle trafficking or biogenesis. Bicaudal D (BICD; Q712J2) has no distinct functional domain. However, homologues proteins of Bicaudal D (Bicaudal D1 and 2) could bind to the small GTPase, Rab6, as well as to dynein-dynactin complexes indicating that it may recruit dynein motor to Rab6-positive membranes of the Golgi apparatus and cytoplasmic vesicles.[56-58] RAB3A-interacting protein (Q62739) has Sec2p domain, a GDP-GTP exchange factor, and endoplasmic reticulum targeting domain. Since Sec2p is involved in vesicular transport at the post-Golgi stage[59] and interacts with small GTPases such as Rab3A, which is associated with secretary vesicle membranes and regulates exocytosis,[60] RAB3A-interacting protein may be involved in the control of vesicular secretion, transport and exocytosis. Neuroplastin (P97546) is a type I transmembrane protein containing immunoglobulin like domain. It has been identified as a major component of synaptic membranes from rat forebrain, and implicated to have an essential role in implementing long-term changes in synaptic activity, possibly by means of a homophilic adhesion mechanism.[61] More biochemical and genetic analysis would reveal the precise function of these proteins.

Complementation of cRPLC/MS-MS and 2DE/MALDI-TOF-MS of proteome analysis. Although there are only a few proteins commonly identified by both proteome analysis methods in this study, other investigations also revealed that 2DE coupled with MALDI-TOF-MS and chromatography coupled with ESI-MS/MS usually identified complementary sets of proteins from the same sample source.[62,63] It is widely accepted that no single analytical approach will identify all the major proteins in any proteome.[63] It is basically due to the difference of ionization mode. In MALDI, almost all peptides are singly-charged, while in ESI longer peptides usually involve doubly or triply-charged.[64-66] Furthermore the ESI analysis includes only those peptides definitively identified *via* MS/MS fragmentation, whereas the MALDI identification is by mass alone.[64]

In summary, we have analyzed proteins from rat brain synaptic vesicles using two different separation methods and mass analysis techniques, cRPLC/MS/MS and 2DE-MALDI-TOF/MS. Most of the identified proteins from each method are not overlap indicating that the efficiency of protein identification varies depending on the extraction and separation methods. This observation suggested that different proteome analysis methods should be applied to identify maximum number of proteins in tissue sample. Among the 27 newly identified proteins in this study, 9 proteins are functionally unknown. Three of the functionally unknown proteins are estimated to involve in trafficking or biogenesis of synaptic vesicles. Further biochemical or genetic analysis of these proteins would reveal their cellular function in synaptic vesicles.

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