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# 2D-NanoLC-ESI-MS/MS for Separation and Identification of Mouse Brain Membrane Proteins

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Phan Van Chi and Nguyen Tien Dung

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/47479>

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## 1. Introduction

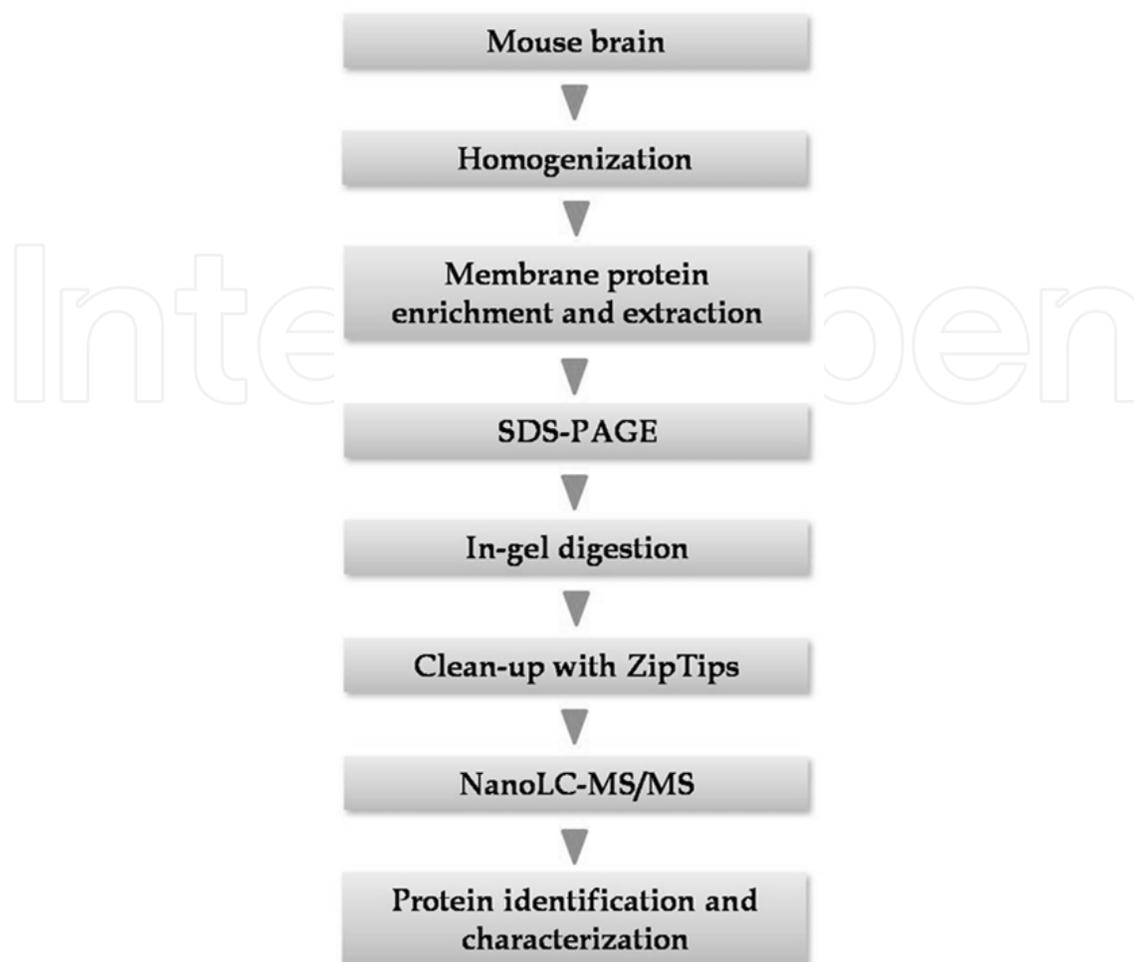
Comprehensive proteomics analysis has the potential to provide new knowledge on cellular responses in development, aging, drug action, environmental stress, and disease pathogenesis (carcinogenesis, cardiovascular disease, etc). However, the separation and identification of proteomes/proteins is a challenging task due to their heterogeneous constituents or complex structures and closely related physico-chemical behaviors. It is clear that the combination of many analytical techniques is necessary to fulfill this complex task. At the start of proteomics research, two-dimensional electrophoresis (2DE) was routinely used to separate complex proteomic sample because of its high resolving power. In this technique, proteins are separated in a two-step process (two dimensions) based on their different physical properties. The first dimension is isoelectrofocusing in which proteins are separated based on their isoelectric points (pI, the pH where a *protein's net charge* is zero) using immobilized pH-gradient strips. Proteins then are separated according to their mass using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. With 2DE, thousands of proteins can be detected in a single experiment depending on the used staining techniques (Coomassie blue, silver, fluorescent dyes staining) [11]. Mass spectrometry (MS), using either electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), is the key technology for the identification of protein spots including membrane proteins, for which differential expression has been demonstrated [16, 30].

2DE, however, has some major drawbacks/disadvantages. It is time-consuming, difficult to reproduce and automation is hard to achieve. Furthermore, 2DE faces with many difficulties in analyzing several groups of proteins, such as low-abundance proteins, hydrophobic

proteins (membrane proteins/membrane-bound and membrane-associated proteins), very large as well as very small proteins and proteins with extreme pI values. Unfortunately, these proteins have high proportion in comparison to total cellular proteins and are usually the most promising targets for drug development or disease diagnostics. About 30% of the mammalian genome encodes integral membrane proteins [27]. However, the comprehensive proteomic analysis of these proteins by mass spectrometry is difficult due to the amphipathic (containing regions that are hydrophobic and hydrophilic) nature in integral membrane proteins and their general low abundance levels [23]. Since the analysis of membrane proteins remains a significant challenge in proteomics, other techniques need to be established to address these problems. There have been many strategies developed for enriching, isolating and separating membrane proteins for proteomic analysis that have moved this field forward.

In recent years, two-dimensional liquid chromatography (2D-LC) has been employed as a complementary or alternative separation technique to 2DE. The combination of liquid chromatography as a separation tool for proteins and peptides with tandem mass spectrometry as an identification tool referred to as LC-MS/MS has generated a powerful and broadly used technique in the field of proteomics [6, 9, 10, 21, 22], particularly in the analysis of membrane proteomes [18, 19]. With the development of new quantitative strategies and bioinformatics tools to cope with the analysis of the large amounts of data generated in proteomics experiments, the resolution and sensitivity state-of-the-art LC-MS/MS systems has reached dimensions allowing not only the analysis of individual proteins but also investigations on the level of complete proteomes [8]. This approach is usually based on the injection of the digested protein sample onto a strong cation-exchange (SCX) column as a first-dimension separation. Peptides bound in SCX column are eluted and separated from the column as fractions by an injecting salt plugs/salt step gradient of increasing salt concentration. Each fraction is subsequently separated on a reversed-phase (RP) column as the second orthogonal separation dimension before being presented to mass spectrometry analysis. Different stationary phases in chromatography columns provide variable levels of resolution. Reversed-phase chromatography is highly compatible with subsequent mass spectrometric analysis due to the lack of salts in the buffers and provides relatively high-resolution separation. Most reversed-phase stationary phases for LC-MS analysis consist of silica beads of 3–5  $\mu\text{m}$  in diameter with alkyl chains of either eight or eighteen carbons in length (C8 or C18) attached. Using column switching, the entire procedure is on-line and fully automated. In order to improve sensitivity the reversed phase separation is usually performed in the nanoflow scale and mass spectrometry is used as the final detection method.

In this chapter a strategy for enrichment, isolation, separation, identification and characterization of mouse brain membrane proteins with the basic *setup* of two-dimensional nano liquid chromatography (2D-nanoLC) system (UltiMate™/FAMOS/Switchos™, LC Parking, Dionex, The Netherlands) coupled online with QSTAR®XL MS/MS mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada) is presented.



**Figure 1.** A scheme illustrating the necessary steps, including enrichment and extraction, separation, identification and characterization for proteomic analyses of mouse brain membrane proteins using gel-based approach in combination with comprehensive two-dimensional nano liquid chromatography (2D-nanoLC) coupled online with tandem mass spectrometry.

## 2. Membrane protein enrichment and extraction

Swiss mouse brains were collected as soon as possible after the animals were killed. The samples (3-5 g) were excised into approximately 5 mm wide pieces using scissors and washed with 10 ml of ice cold PBS buffer (0.2 g KCl, 8 g NaCl, 1.44 Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g KH<sub>2</sub>PO<sub>4</sub>) and then resuspended in 3 volumes of the homogenization medium (0.25 M sucrose in 5 mM Tris-HCl pH 7.4 with 1 mM tetrasodium EGTA, 1 mM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>) and 2 mM sodium fluoride in deionized filter-sterilised MilliQ water) containing protease inhibitors (Calbiochem Protease Inhibitor Cocktail Set 111, catalog number 39134, contains AEBSF, aprotinin, bestatin, E-64, leupeptin, pepstatin A). After the medium has been drained off, new medium was replaced and drained off again. 10 ml of homogenisation medium (containing inhibitors) was added and the sample was homogenised using a Polytron in a Potter homogeniser with motor driven teflon pestle at approximately 1,000 rpm. Completely homogenized samples were

centrifuged at 10,000 rpm for 15 min at 4°C to sediment large organelles. The obtained supernatant was used for recentrifugation again at 10,000 rpm for 15 min at 4°C. The supernatant was collected and centrifuged at 40,000 rpm at 4°C for 1 hr. After discarding the clear supernatant, the membrane pellets were retained and washed by resuspending in ice-cold 0.1 M Na<sub>2</sub>CO<sub>3</sub> containing protease inhibitors for 1 hr. The mouse brain membrane protein fractions were obtained by centrifugation again at 40,000 rpm for 1 h at 4°C. The sample was divided and stored at -80°C until use. The protein concentration of the extracted membrane fractions was assessed using a Quick Start™ Bradford Protein Assay Kit (Bio-Rad, Hercules, CA 94547 USA).

### 3. Protein quantification

Protein concentration of the extracted membrane fractions was determined using Bio-Rad's Quick Start™ Bradford Protein Assay [5]. The assay is based on the observation that the maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible colour change. For the standard curve, bovine serum albumin over a wide range of concentrations (0.1 - 20 µg/µl) was used. The low concentration range assay was used in the test tube format. 2 µl of standard or sample was added to 798 µl of MilliQ water. 200 µL of Bio-Rad reagent was added, mixed, and incubated for 10 min at room temperature. The absorbance at the wavelength of 595 nm was measured in a spectrophotometer. Glass or polystyrene (cheap) cuvettes have been used, however the color reagent stained both. Disposable cuvettes were recommended.

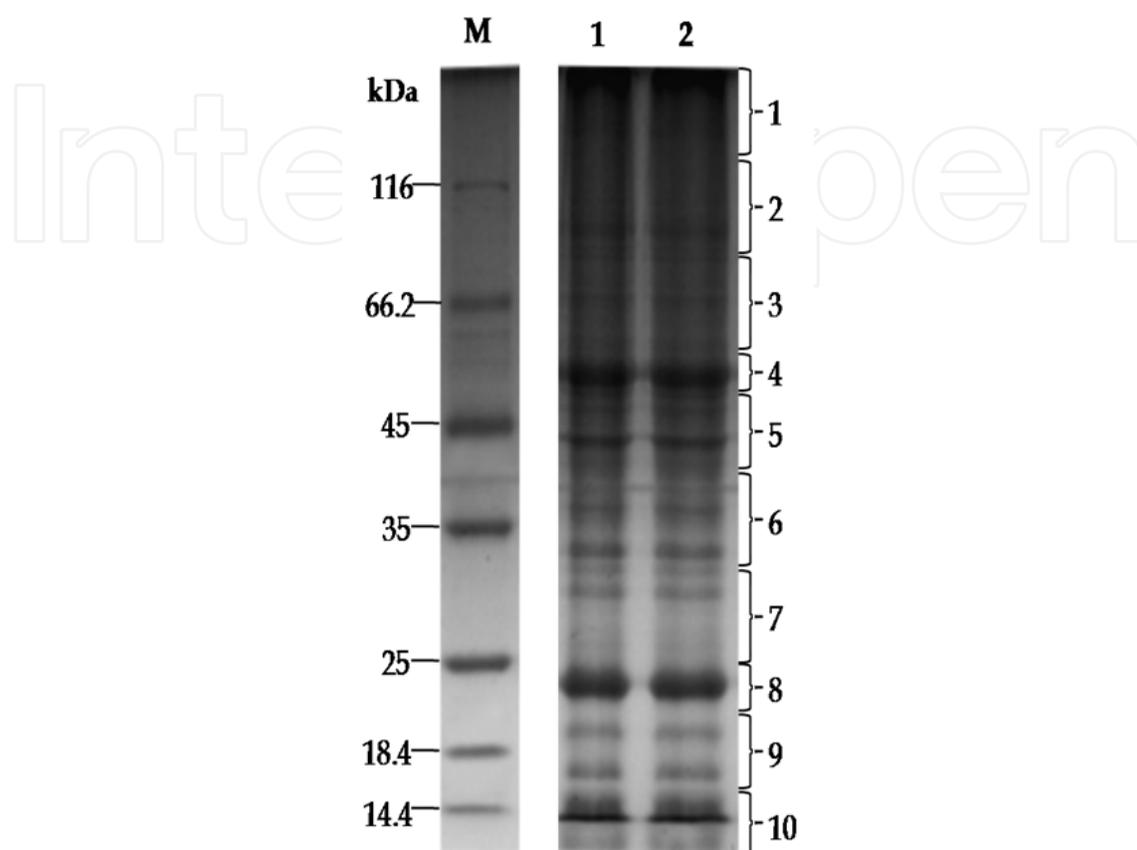
## 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel digestion

### 4.1. SDS-PAGE

All chemicals including Precision Plus Protein Unstained Standards (catalog number: 161-0363EDU), Coomassie Brilliant Blue G-250 Staining Solution Kit (catalog number: 161-0435EDU) used for SDS-PAGE, were purchased from Bio-Rad (Bio-Rad Laboratories, Inc., CA 94547, USA). The procedure was carried out according to Laemmli [14].

The following stock solutions were prepared: (i) 1.5 M Tris-HCl, pH 8.8; (ii) 0.5 M Tris-HCl, pH 6.8; (iii) 30% acrylamide/bisacrylamide solution (37.5:1); (iv) *N,N,N,N*-tetramethylethylenediamine (TEMED); (v) freshly prepared 10% ammonium persulphate (APS) solution; (vi) 10% sodium dodecyl sulphate (SDS) solution; (vii) 10X SDS gel running buffer (30 g Tris-base, 144 g glycine, 10 g SDS, dissolved in MilliQ water and adjusted to a volume of one liter); (viii) 5X sample buffer (10% SDS, 50% glycerol, 300 mM Tris-HCl (pH 6.8), 0.05% bromphenol blue. Dithiothreitol (DTT) was added to a final concentration of 100 mM prior to use).

The membrane fraction was solubilized in lysis buffer containing 3% SDS. Equal volumes containing approximately 25  $\mu\text{g}/\text{lane}$  of MP were separated by 12% SDS-PAGE and were visualized by staining with Coomassie Brilliant Blue G-250.



**Figure 2.** The separation of membrane proteins (MPs) by SDS-PAGE. The gel was cut into 10 slices that covered known apparent mass ranges. Lane M, protein standard markers; lane 1 & lane 2: membrane protein fractions isolated from mouse brain; 1-10: slices to be cut for trypsin in-gel digestion, separation and analyses by nanoLC-MS/MS.

#### 4.2. In-gel digestion

In-gel digestion of proteins isolated by gel electrophoresis was carried out according to the protocol published by Shevchenko *et al* [25] with some modifications described in our previous study [3, 28, 29]. All chemicals including DTT, iodoacetamide (IAA), ammonium bicarbonate, ammonium acetate, trypsin (proteomics sequencing grade), sodium bicarbonate and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA) prepared using deionized filter-sterilised MilliQ water.

Upon electrophoresis, proteins were fixed within a polyacrylamide matrix by incubating the entire gel in 5% (vol/vol) acetic acid in 1:1 (vol/vol) water:methanol. Coomassie blue-stained protein bands were excised from gels and placed into 1.5 ml eppendorf tubes, destained with 50% ACN in 25 mM  $\text{NH}_4\text{HCO}_3$  pH 8.0 at room temperature with occasional vortexing, until gel pieces became white and shrank, and then acetonitrile was removed. The gel pieces

were then reduced by incubating with 5 mM DTT solution at 56°C for 45 min and alkylated for 1 hr with 20 mM IAA solution in darkness at room temperature. The membrane proteins were digested by adding trypsin buffer (0.03 µg/µl in 10 mM ammonium bicarbonate containing 10% (vol/vol) acetonitrile) and incubating overnight at 37°C. Check if all solution was absorbed and add more trypsin buffer, if necessary. Gel pieces should be completely covered with trypsin buffer (typically, 50 µl or more).

### 4.3. Sample cleanup with C-18 ZipTips

The resulting peptide digestion products were extracted by adding 100 µl of extraction buffer (1:2 (vol/vol) 5% formic acid/acetonitrile) to each tube and incubated for 15 min at 37 °C in a shaker. All extracts were saved and dried and re-dissolved in 10–20 µl of 0.1% FA, incubated for 2–5 min in the sonication bath and centrifuged for 15 min at 10,000 rpm at the bench-top centrifuge. The obtained supernatant was applied for binding the samples onto micro pipette tips (µC18), catalog number ZTC18S096 (ZipTip®, Millipore Co., Billerica, MA 01821 USA), equilibrated by being aspirated and dispensed with 100% acetonitrile, 40% acetonitrile/0.1% FA, 0.1% FA solutions. The samples were washed (4 times by aspirating and dispensing) with 15 µl of 0.1% FA, then eluted with 10 µl of 40% acetonitrile/0.1% FA. Appropriate aliquots were withdrawn for LC-MS/MS analysis or store at –20°C as contingency.

## 5. Two-dimensional nano liquid chromatography (2D-nanoLC)

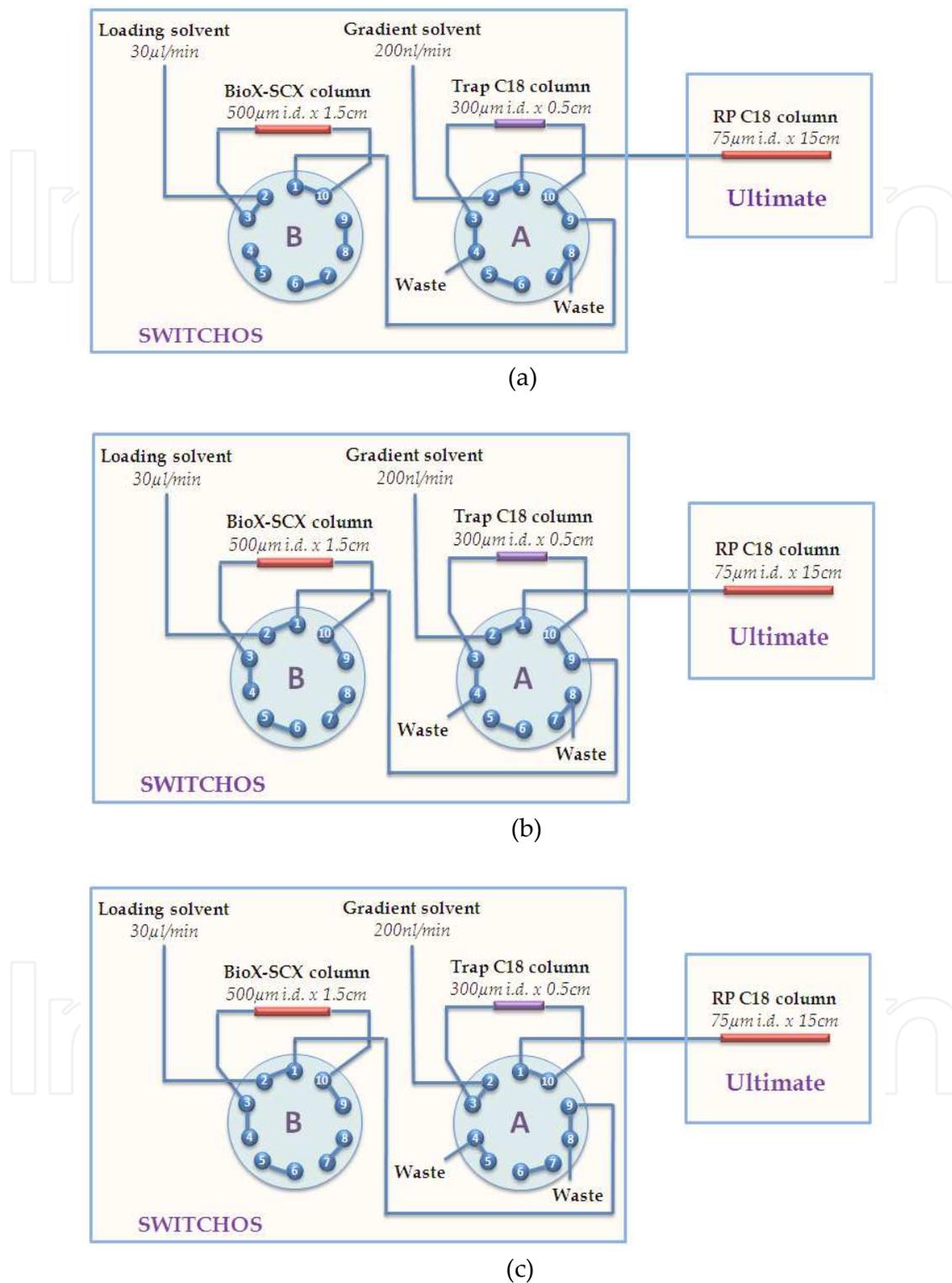
The basic setup of an online two-dimensional nano liquid chromatography (2D-nanoLC) system (LC Parking, Dionex, The Netherlands) was developed for improved separation and hydrophobic peptide recovery, especially for complex peptides made from enzymatic digests of selected proteomes. The system works with the principle of elution of the digested peptides from the first dimension SCX column with injected salt solution plugs of increasing concentration. The eluted peptides are again trapped and introduced into the nanoflow path for separation and analysis by second dimension RP column and tandem mass spectrometry. The great advantage of the system is a robust and fully automated separation. The methods are easy to set up and composed of identical runs differing only in the concentration of injected salt plugs.

For the mentioned above online 2D-nanoLC system, the following columns were used: (i) strong cation exchange (SCX) column (500 µm i.d.×1.5 cm) packed with BioX-SCX, 300 Å, 5 µm, (LC Parking, Dionex, P/N 161395); (ii) Trap column: 300 µm i.d.× 0.5 cm, packed with PepMap™ C18, 100 Å, 5 µm, (LC Parking, Dionex, P/N 160454); (iii) Reversed phase (RP) column: 75 µm i.d.× 15 cm, packed with C18 PepMap100, 100 Å, 3 µm, (LC Parking, Dionex, P/N 160321). The column physico-chemical properties, functions, and the mobile phase, loading/eluted solvents for the flow diagram in online 2D-nanoLC system (UltiMate™/FAMOS/Switchos™, LC Packings, Dionex) with the 10-port valve automatic switching configuration are shown in details in table 1 and figure 3.

	1	2	3	4
Type of column		1 <sup>st</sup> Dimension - Exchange Ion Chromatography- SCX column	PepMap nano RP Trapping column	2 <sup>nd</sup> Dimension- Reversed Phase C18 column
Function		Separation of a protein/peptide on an <i>ion exchange</i>	Pre-concentrating sample	Separation of a protein/peptide on a reversed phase
Physical properties of column		500 $\mu\text{m}$ i.d. x 1.5 cm, 300 $\text{\AA}$ , 5 $\mu\text{m}$ (LC Parking, Dionex, P/N 161395)	300 $\mu\text{m}$ i.d. x 0.5 cm, PepMap <sup>TM</sup> C18, 5 $\mu\text{m}$ , 100 $\text{\AA}$ (LC Parking, Dionex, P/N 160454)	75 $\mu\text{m}$ i.d. x 15 cm, packed with PepMap <sup>TM</sup> C18, 100 $\text{\AA}$ , 3 $\mu\text{m}$ , (LC Parking, Dionex, P/N 160321)
Injected volumn		20 $\mu\text{l}$		
Flow rate		200 nl/min		
Loading flow			30 $\mu\text{l}/\text{min}$	
Loading solvent			0.1% FA, pH2.9	
Eluted solvent (Elute positively charged peptides on SCX)		Ammonium acetate solutions: 10 mM, 20 mM, 40mM, 60mM, 80mM, 100 mM, 200 mM, 500mM, 1M, 2M		
Mobile phase				A (0.1% FA in LC-MS grade water); B (0.1% FA in 85% LC-MS grade ACN)

**Table 1.** The type of columns with their physico-chemical properties, functions and the mobile phase, loading/eluted solvents that were used for basic experimental setup of an online two-dimensional nano liquid chromatography system (2D-nanoLC, UltiMate<sup>TM</sup>/FAMOS/Switchos<sup>TM</sup>, LC Parking, Dionex, The Netherlands).

For in-gel digest samples, as the first step, the peptide mixture was re-dissolved in 30  $\mu\text{l}$  of 0.1% FA and directly loaded onto a strong cation exchange (SCX) column (500  $\mu\text{m}$  i.d. x 1.5 cm, 5  $\mu\text{m}$ , 300  $\text{\AA}$ ) at a flow rate of 30  $\mu\text{l}/\text{min}$ . Bound peptides were eluted by following ammonium acetate gradients from 10 mM to 2 M: 10 mM, 20 mM, 40mM, 60mM, 80mM, 100 mM, 200 mM, 500mM, 1M and 2M and then desalted and concentrated independently on a C18 trap column (300  $\mu\text{m}$  i.d. x 0.5 cm, 5  $\mu\text{m}$ , 100  $\text{\AA}$ ). The eluted peptides were further separated onto a reversed phase C18 column (75  $\mu\text{m}$  i.d. x 15 cm, 5  $\mu\text{m}$ , 100  $\text{\AA}$ ), for the second dimension. The flow rate was maintained at 200 nl/min with solvent A (0.1% FA in LC-MS grade water). With 10 different concentrations of ammonium acetate (plugs), there should be 10 identical runs.



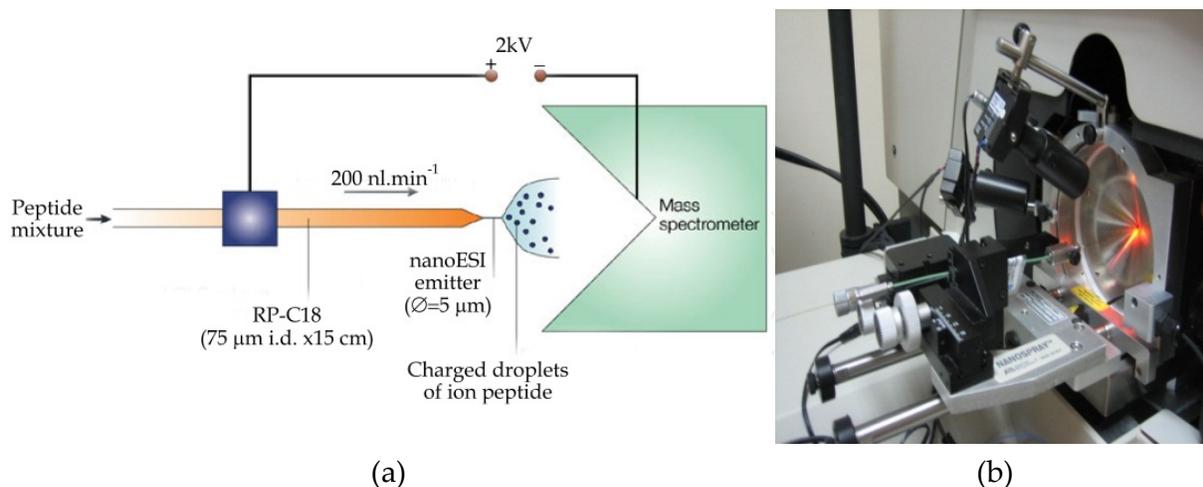
**Figure 3.** Illustration of the flow diagram in online 2D-nanoLC system (UltiMate™/FAMOS/Switchos™, LC Packings, Dionex) with the 10-port valve automatic switching configuration and localization of BioX-SCX column, Trap C18 column and RP C18 column in: (a) loading mode, (b) clean-up mode and (c) analysis mode.

After washing (~12 min), peptides were eluted from a reversed phase C18 column using the solvent B (0.1% FA in 85% LC-MS grade ACN) gradients: from 5 to 20% of solvent B in 25 min, 20 to 70% in 28 min, 70 to 100% in 10 min and maintaining 100% B in 10 min, and back to 5% B in 5 min.

## 6. Integrating NanoLC system and tandem mass spectrometer

In our example, samples were delivered into the instrument by an automated in-line (integrated LC Parking's System, 5 mm C18 nano-precursor and 75  $\mu\text{m}$  i.d.  $\times$  15 cm column, packed with C18 PepMap100, 100  $\text{\AA}$ , 3  $\mu\text{m}$ , (LC Parking, Dionex, P/N 160321) via a nanoelectrospray source head and 10  $\mu\text{m}$  inner diameter PicoTip (New Objective, Massachusetts, USA) (Figure 4).

According to the workflow, after 2D-nanoLC separation, peptides were independently analyzed by a QSTAR<sup>®</sup>XL MS/MS mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada) equipped with a nanoESI source. MS and MS/MS spectra were recorded and processed in IDA mode (Information Dependent Acquisition) controlled by Analyst QS software. Typical settings are chosen to select multiply charged ions for MS/MS that produce at least 45-50 ion counts/s in a 0.5 s survey scan. The range of the MS full scan was from 400 to 1200 amu followed by MS/MS fragmentation of the three most intense precursor peptide ions for 1 s each.



**Figure 4.** Setup and demonstration of nanoLC-MS interface, link between nanoLC with nanoelectrospray ionization source and tandem mass spectrometry: (a) Schematic diagram of a nanoLC-MS interface; (b) Interface of nanoLC (LC Parkings, Dionex, Netherlands) with QSTAR<sup>®</sup>XL MS/MS mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada). By using this system, complex sample can be injected, desalted, separated and analyzed in complete automatization.

## 7. Protein identification and validation

There are a number of different methods for identifying the proteins in the sample, and the most frequently used is the searching of the uninterpreted MS/MS data. The FASTA formatted protein sequences from National Center for BioTechnology Information (NCBI) and UniProtKB/Swiss-Prot databases are collected for proteins identified or identification by each MS experiment. Searching uninterpreted MS/MS data from a single peptide or from a complete nanoLC-MS/MS run was automatically analyzed with a non-redundant protein database by the program SEQUEST, which allows the correlation of experimental data with theoretical spectra generated from known protein sequences [7].

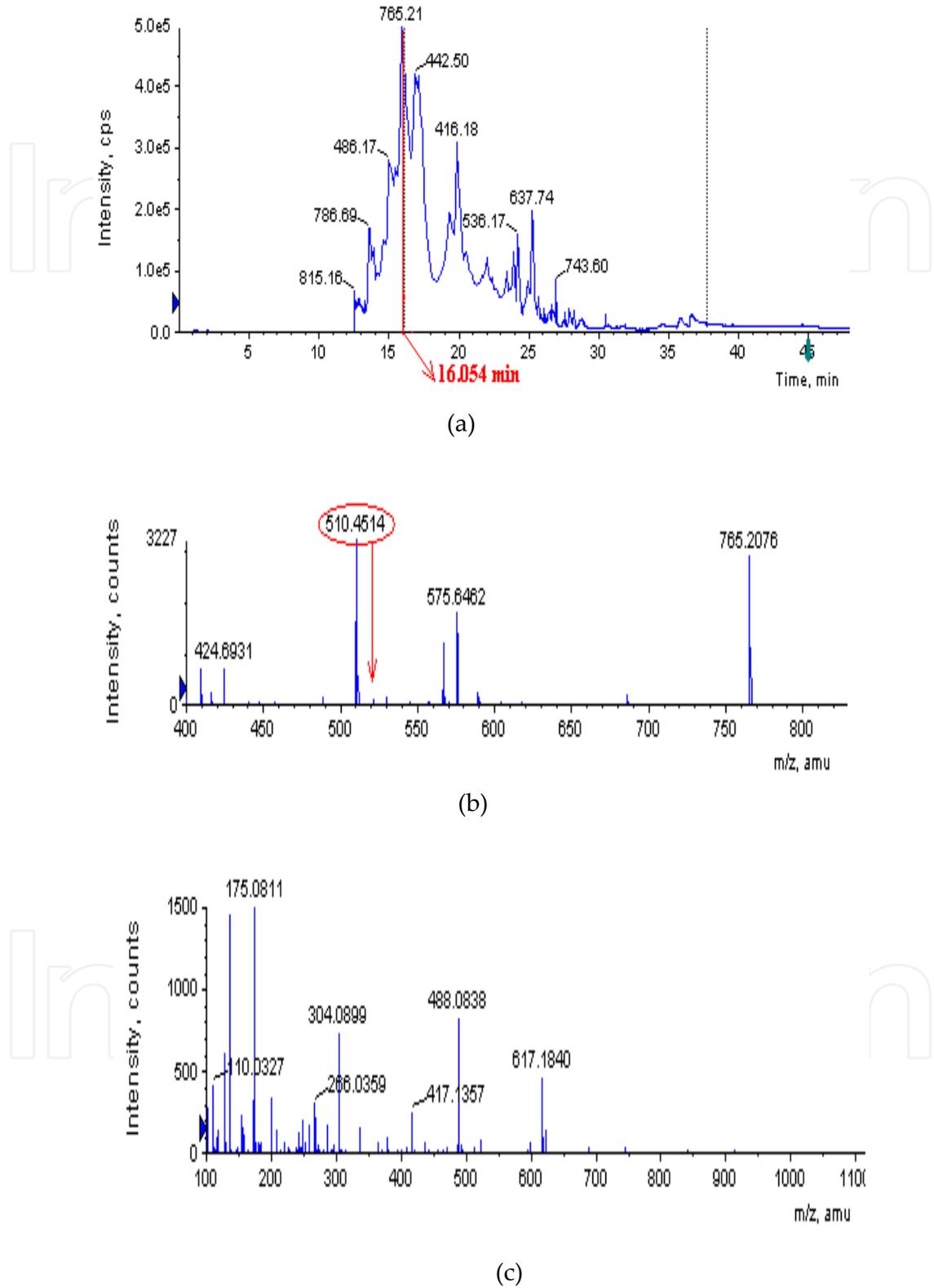
The precursor mass is used as a filter to find a list of candidate peptide sequences from the theoretical digest of the database. A variety of different systems are used to score the experimental MS/MS spectrum against spectra predicted from the candidate peptide sequences. For protein identification, experimental data were searched against the NCBI and Swiss-Prot mouse protein database using Mascot v1.8 software in which the criteria were based on the manufacturer's definitions (Matrix Science Ltd, London, UK) [20]. The parameters were set as follows: enzymatic cleavage with trypsin; 1 potential missed cleavage; a peptide and fragment mass tolerance of  $\pm 0.25$  Da, and fixed modification of carbamidomethyl (cysteine); variable modification of oxidation (methionine); 1<sup>+</sup>, 2<sup>+</sup>, and 3<sup>+</sup> peptide charge. Protein identifications were performed using a Mowse scoring algorithm with a confidence level of 95% and at least two matched peptides, showing a high score [12].

For further verification, proteins might be validated by MSQuant software [1, 4, 24] available at <http://msquant.sourceforge.net>. The MSQuant software is used as a validation and quantitation tool that produces the Mascot peptide identifications (HTML files) and allows manual verification against the raw MS data (QSTAR XL raw files). The MSQuant software will pick up significant and verified hits from the Mascot output file and export information of identified proteins into an .xls file, including the GI (genInfo identifier) number and molecular-mass values.

## 8. Prediction of transmembrane domains (TMDs)

The identified proteins were categorized based on their cellular locations and biological processes according to Gene Ontology (GO) information obtained at <http://www.ebi.ac.uk/pub/databases/GO/goa/mouse> and <ftp://ftp.geneontology.org/pub/go/> [2]. The TMHMM ([www.cbs.dtu.dk/services/TMHMM/](http://www.cbs.dtu.dk/services/TMHMM/)) algorithm was used to predict transmembrane domains (TMDs) [15, 26].

The average hydrophobic values and transmembrane domains of the identified proteins were calculated using the SOSUI system that is available at <http://bp.nuap.nagoya-u.ac.jp/sosui/> [11]. The proteins exhibiting positive GRAVY values were recognized as hydrophobic and those with negative values were hydrophilic [13].



**Figure 5.** Illustration of 2D-nanoLC-ESI-MS/MS spectra: (a) The total ion current (TIC) profile tryptic digest of membrane proteins (band 6) at the concentration of 100 mM ammonium acetate for run time 0-50 min; (b) TOF-MS spectrum at 16.054 min; (c) TOF product spectrum of a peptide ion with  $m/z = 510.45$ .

Peptide Summary Report (Mou x)

D:\PROJECTS\NASFOTED\MASCOT RESULT\B6

**Mascot Search Results**

**User** : Nguyen Tien Dung  
**Email** : nguyentienung\_bk@yahoo.com  
**Search title** : Mouse membrane - Band 6  
**MS data file** : CM6D\_CID.pk1  
**Database** : SwissProt 2011 (533049 sequences; 189064225 residues)  
**Taxonomy** : Mus musculus (house mouse) (16480 sequences)  
**Timestamp** : 15 Dec 2011 at 10:25:38 GMT

**Protein hits** :

- [ADT2\\_MOUSE](#) ADP/ATP translocase 2 OS=Mus musculus GN=Slc25a5 PE=1 SV=3
- [MOG\\_MOUSE](#) Myelin-oligodendrocyte glycoprotein OS=Mus musculus GN=Mog PE=1 SV=1
- [PHB\\_MOUSE](#) Prohibitin OS=Mus musculus GN=Phb PE=1 SV=1
- [NDUS3\\_MOUSE](#) NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial
- [ADT1\\_MOUSE](#) ADP/ATP translocase 1 OS=Mus musculus GN=Slc25a4 PE=1 SV=4
- [VDAC1\\_MOUSE](#) Voltage-dependent anion-selective channel protein 1 OS=Mus musculus
- [TBA1A\\_MOUSE](#) Tubulin alpha-1A chain OS=Mus musculus GN=Tuba1a PE=1 SV=1
- [VDAC2\\_MOUSE](#) Voltage-dependent anion-selective channel protein 2 OS=Mus musculus
- [TBB2A\\_MOUSE](#) Tubulin beta-2A chain OS=Mus musculus GN=Tubb2a PE=1 SV=1
- [M20M\\_MOUSE](#) Mitochondrial 2-oxoglutarate/malate carrier protein OS=Mus musculus
- [MPCP\\_MOUSE](#) Phosphate carrier protein, mitochondrial OS=Mus musculus GN=Slc25a3
- [TBB5\\_MOUSE](#) Tubulin beta-5 chain OS=Mus musculus GN=Tubb5 PE=1 SV=1
- [TBB4B\\_MOUSE](#) Tubulin beta-4B chain OS=Mus musculus GN=Tubb4b PE=1 SV=1
- [TBB3\\_MOUSE](#) Tubulin beta-3 chain OS=Mus musculus GN=Tubb3 PE=1 SV=1
- [ATPG\\_MOUSE](#) ATP synthase subunit gamma, mitochondrial OS=Mus musculus GN=Atp5c1
- [CY1\\_MOUSE](#) Cytochrome c1, heme protein, mitochondrial OS=Mus musculus GN=Cyc1
- [TBB6\\_MOUSE](#) Tubulin beta-6 chain OS=Mus musculus GN=Tubb6 PE=1 SV=1
- [TBA4A\\_MOUSE](#) Tubulin alpha-4A chain OS=Mus musculus GN=Tuba4a PE=1 SV=1
- [NSF\\_MOUSE](#) Vesicle-fusing ATPase OS=Mus musculus GN=Nsf PE=1 SV=2
- [VDAC3\\_MOUSE](#) Voltage-dependent anion-selective channel protein 3 OS=Mus musculus
- [H12\\_MOUSE](#) Histone H1.2 OS=Mus musculus GN=Hist1hc PE=1 SV=2
- [CN37\\_MOUSE](#) 2',3'-cyclic-nucleotide 3'-phosphodiesterase OS=Mus musculus GN=Cnp
- [1433E\\_MOUSE](#) 14-3-3 protein epsilon OS=Mus musculus GN=Ywhae PE=1 SV=1
- [KCC2A\\_MOUSE](#) Calcium/calmodulin-dependent protein kinase type II subunit alpha
- [ETFB\\_MOUSE](#) Electron transfer flavoprotein subunit beta OS=Mus musculus GN=Etfb
- [1433G\\_MOUSE](#) 14-3-3 protein gamma OS=Mus musculus GN=Ywhag PE=1 SV=2
- [AINX\\_MOUSE](#) Alpha-internexin OS=Mus musculus GN=Ina PE=1 SV=2
- [RAB3A\\_MOUSE](#) Ras-related protein Rab-3A OS=Mus musculus GN=Rab3a PE=1 SV=1
- [MBP\\_MOUSE](#) Myelin basic protein OS=Mus musculus GN=Mbp PE=1 SV=2
- [1433T\\_MOUSE](#) 14-3-3 protein theta OS=Mus musculus GN=Ywhaq PE=1 SV=1
- [GSTM1\\_MOUSE](#) Glutathione S-transferase Mu 1 OS=Mus musculus GN=Gstm1 PE=1 SV=2
- [PHB2\\_MOUSE](#) Prohibitin-2 OS=Mus musculus GN=Phb2 PE=1 SV=1
- [NFL\\_MOUSE](#) Neurofilament light polypeptide OS=Mus musculus GN=Nefl PE=1 SV=5
- [GFAP\\_MOUSE](#) Glial fibrillary acidic protein OS=Mus musculus GN=Gfap PE=1 SV=4
- [1433Z\\_MOUSE](#) 14-3-3 protein zeta/delta OS=Mus musculus GN=Ywhaz PE=1 SV=1
- [MYPR\\_MOUSE](#) Myelin proteolipid protein OS=Mus musculus GN=Plp1 PE=1 SV=2
- [H14\\_MOUSE](#) Histone H1.4 OS=Mus musculus GN=Hist1hle PE=1 SV=2
- [NFH\\_MOUSE](#) Neurofilament heavy polypeptide OS=Mus musculus GN=Nefh PE=1 SV=3

**Figure 6.** An example of Mascot search result shows list of the identified mouse brain membrane proteins isolated from band 6 (see figure 2) and their accession numbers, using SwissProt database (533049 sequences, 189064225 residues)



## SOSUI Result

Query title : None

Total length : 303 A. A.

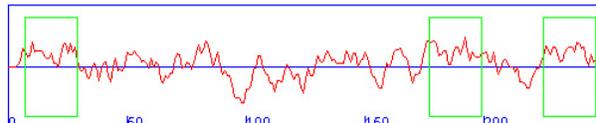
Average of hydrophobicity : 0.250825

**This amino acid sequence is of a MEMBRANE PROTEIN  
which have 3 transmembrane helices.**

No.	N terminal	transmembrane region	C terminal	type	length
1	7	QVLLGSGLTILSQPLMYVKVLIQ	29	SECONDARY	23
2	177	GFFAGLIPRLGDIISLWLCNSL	199	PRIMARY	23
3	225	VTGFFASMLTYPFVLVSNLMAVN	247	SECONDARY	23

## Display Options

### [Hydropathy profile]



### [Helical wheel diagram of predicted segments]

**Figure 7.** An example of hydropathy profile and transmembrane regions/domains of an identified mouse brain membrane protein calculated using the SOSUI system that is available at <http://bp.nuap.nagoya-u.ac.jp/sosui/> [11].

## 9. Conclusion

Identification and characterization of membrane proteins is a crucial challenge in proteomics research. Thus, we have designed a strategy of gel-based approach in combination with comprehensive two-dimensional nano liquid chromatography (2D-nanoLC) that is robust and offers high separation capacity and high analysis throughput for mouse brain membrane proteins. By using this system, mixtures of in-gel trypsin-digested mouse brain membrane proteins were injected, desalted, separated and analyzed in complete automatization. The workflow started by the extraction and purification of the membrane fractions, then the SDS-PAGE was carried out as a useful preparative separation step. After staining, the gel slides with protein bands were cut, reduced, alkylated and

trypsin-digested. The peptide mixtures extracted from each gel slice were fractionated by 2D-nanoLC coupled online with tandem mass spectrometry analysis (nanoESI-Q-TOF-MS/MS). The proteins were identified by MASCOT search against mouse protein database using a peptide and fragment mass tolerance of  $\pm 0.25$  Da. Protein identification was carried out using a MOWSE scoring algorithm with a confidence level of 95% and processed by MSQuant software for further validation. In total, 298 identified membrane proteins from mouse brain tissues were verified by UniProt database, SOSUI and TMHMM prediction algorithms. Of these, 129 (43.3%) proteins have at least one transmembrane domain according to SOSUI and TMHMM. Furthermore, the function, subcellular location, molecular weight, post-translational modifications, transmembrane domains (TMD) and average of hydrophobicity of the identified membrane proteins might be categorized and analysed.

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