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Proteomic analysis of rat hippocampal plasma membrane: characterization of potential neuronal-specific plasma membrane proteins

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Abstract

The hippocampus is a distinct brain structure that is crucial in memory storage and retrieval. To identify comprehensively proteins of hippocampal plasma membrane (PM) and detect the neuronal-specific PM proteins, we performed a proteomic analysis of rat hippocampus PM using the following three technical strategies. First, proteins of the PM were purified by differential and density-gradient centrifugation from hippocampal tissue and separated by one-dimensional electophoresis, digested with trypsin and analyzed by electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) tandem mass spectrometry (MS/MS). Second, the tryptic peptide mixture from PMs purified from hippocampal tissue using the centrifugation method was analyzed by liquid chromatography ion-trap ESI-MS/MS. Finally, the PM proteins from primary hippocampal neurons purified by a biotin-directed affinity technique were separated by one-dimensional electrophoresis, digested with trypsin and analyzed by ESI-Q-TOF-MS/MS. A total of 345, 452 and 336 non-redundant proteins were identified by each technical procedure respectively. There was a total of 867 non-redundant protein entries, of which 64.9% are integral membrane or membrane-associated proteins. One hundred and eighty-one proteins were detected only in the primary neurons and could be regarded as neuronal PM marker candidates. We also found some hypothetical proteins with no functional annotations that were first found in the hippocampal PM. This work will pave the way for further elucidation of the mechanisms of hippocampal function.

Keywords: affinity purification, hippocampus, mass spectrometry, plasma membrane, primary neuron, proteomics.
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The plasma membrane (PM) is a selectively permeable barrier and communication interface of cells owing to the presence of specific membrane proteins, which play important biological and pharmacological roles in intercellular communication, cellular development, cell migration and drug resistance. Indeed, about two-thirds of all drug targets are PM proteins (Hopkins and Groom 2002). With the advent of mass spectrometry (MS)-based proteomics (Aebersold and Mann 2003), it is now possible to profile globally the proteome of an organism, including organelles, tissues, cells or subcellular compartments. Despite the biological importance of these proteins, they seem to be disproportionately undercharacterized from biochemical, topographical and structural perspectives owing to their hydrophobic nature and general low abundance, which are the two major limitations of the conventional proteomic strategy using two-dimensional electophoresis (2DE) as the main protein separation method. Although novel non-ionic or zwitterionic detergents have been used extensively for separation of membrane proteins on 2DE

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Abbreviations used: ACN, aectonitrile, ApoE, apolipoprotein E; APPM, affinity-purified plasma membrane; 2DE, two-dimensional gel electrophoresis; DTT, dithiothreitol; ER, endoplasmic reticulum; ESI, electrospray ionization; FUDR, 5-fluoro-2-deoxyuridine; GO, gene ontology; GRAVY, grand average of hydropathy; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LTQ, linear ion trap quadrupole; Mit, miochondrion; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MW, molecular weight; NF, neurofilament; PBS, phosphate-buffered saline; pI, isoelectric point; PM, plasma membrane; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; Q-TOF, quadrupole time-of-flight; sulfo-NHS-LC-biotin, sulfosuccinimidyl-6-(biotin-amido)hexanoate; sulfo-NHS-SS-biotin, sulfosuccinimidyl-2-(biotinamido)ethyl-1,3 dithiopropionate; RP, reversed-phase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris-buffered saline containing 0.1% Tween-20; TM, transmembrane; TMHMM, transmembrane hidden Markov model; unchar, uncharacterized; Xcorr, cross-correlation.

with immobilized pH gradient strips in the first dimension (Santoni *et al*. 2000), no significant membrane protein separation on 2DE with high dynamic resolution has been reported. Currently the best strategy for analysis of membrane fractions is the combination of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with liquid chromatography (LC) tandem MS (MS/MS), which is termed geLC-MS/MS (Li *et al*. 2003), or the direct gel-free method using shotgun strategies (Washburn *et al*. 2001).

Another problem with comprehensive studies of the PM proteome is the low abundance of many PM proteins. Even with improved sensitivity of MS instruments, membrane proteins are underrepresented in the analysis of whole-cell protein extracts. Many subcellular methods and prefractionation or enrichment approaches have been developed (Dreger 2003; Peltier *et al*. 2004). Typically, PM is isolated by a combination of differential and density-gradient centrifugation, based on density differences between the PM and other subcellular organelles (Emmelot *et al*. 1974). Because the density of the PM is similar to that of mitochondria and endoplasmic reticulum (ER), the membrane fractions prepared by ultracentrifugation methods are often contaminated with these organelles. However, owing to its simplicity and suitability of the procedure for most samples, it is still used for most PM proteome analysis (Adam *et al*. 2003; Stevens *et al*. 2003; Blonder *et al*. 2004). Several advances in the study of the PM subproteome using various membrane purification methods have been described recently (Durr *et al*. 2004; Marmagne *et al*. 2004; Peirce *et al*. 2004; Zhao *et al*. 2004). For example, affinity methods based on cell surface biotinylation and affinity enrichment using immobilized beads or monomeric avidin columns for the isolation of either PM sheets or PM proteins have been widely used (Shin *et al*. 2003; Zhang *et al*. 2003; Gauthier *et al*. 2004; Peirce *et al*. 2004). Such methods appear efficient for analyzing the PM proteome of various cell lines with low contamination and a high level of reproducibility.

The hippocampus is a horseshoe-shaped and cytoarchitecturally distinct structure folded into the cerebral cortex. It plays a central role in learning and memory processes as well as in the stress response. Abnormal protein expression in the hippocampus can result in multiple neurological diseases, such as Huntington's chorea, Alzheimer's disease (Maezawa *et al*. 2004) and Parkinson's disease (Bezard *et al*. 2005). In the past few years, proteomic approaches have been used to profile the human and murine hippocampal proteome in a number of studies (Edgar *et al*. 1999; Yang *et al*. 2004; Fountoulakis *et al*. 2005). At the subcellular level, Nielsen *et al*. developed a gel-free technology for proteomic mapping of mouse brain PM proteins. They identified 862 proteins from mouse brain cortex and 1685 proteins from hippocampus using quadrupole time-of-flight (Q-TOF) MS and linear ion trap quadrupole-Fourier-Transfer (LTQ-FT) MS respectively (Nielsen *et al*. 2005).

All the results so far reported have been generated from tissue from the entire hippocampus or its compartments. It is known that the whole tissue is highly complex and heterogeneous, and the whole cellular proteomes are difficult to be profiled without contaminants. Ideally, a single, defined cell type should be used as starting material, for example clonal neuronal cell lines or primary cultures of neurons and astrocytes (Choudhary and Grant 2004); such defined samples are relatively homogeneous and permit the identification of low-level regulatory components and cell type-specific protein markers.

In this study, PM isolated from hippocampal tissue using centrifugation-based methods was analyzed both by geLC-MS/MS and shotgun strategies. and we also studied affinity-purified PM from primary neurons by geLC-MS/MS. In total, 867 non-redundant putative PM proteins were identified, of which 64.9% are integral membrane or membrane-associated proteins. This analysis also revealed some hypothetical proteins found in hippocampal PM for the first time. This PM protein database of rat hippocampus included 181 proteins that were characterized in the primary neuronal fractions only and could be regarded as neuronal marker candidates. This work extends our knowledge of brain membrane proteome research, and will contribute to our understanding of hippocampus-related neurological functions.

Materials and methods

Materials

EZ-Link sulfosuccinimidyl-2-(biotinamido)ethyl-1,3 dithiopropionate (sulfo-NHS-SS-biotin) and Western Lightning Chemiluminescence Reagent Plus were from Pierce (Rockford, IL, USA). Dynabeads M-280 streptavidin and magnetic plate were obtained from Dynal Biotech ASA (Oslo, Norway). Proteomics sequencing-grade trypsin, endoproteinase Lys-C, dithiothreitol (DTT), iodoacetamide, trifluoroacetic acid, 5-fluoro-2-deoxyuridine (FUDR), HEPES and sucrose were obtained from Sigma-Aldrich (St Louis, MO, USA). Acrylamide, bis-acrylamide, urea, glycine, Tris and SDS were from Amresco (Solon, OH, USA). Immobilon transfer membranes (polyvinylidene difluoride; PVDF) were from Millipore (Bredford, MA, USA). Bio-Rad DC protein assay kit and Sypro Ruby protein blot gel stain were bought from Bio-Rad Laboratories (Hercules, CA, USA). Anti-flotillin-1 (monoclonal) and horseradish peroxidase-conjugated anti-mouse IgG were obtained from BD Bioscience (San Jose, CA, USA). High-performance liquid chromatography (HPLC)-grade acetonitrile and acetone were from China National Medicine Group Shanghai Chemical Reagent Company (Shanghai, China). Fetal bovine serum was bought from Tianjing Blood Institute (Tianjing, China). Water was obtained from a Milli-Q Plus purification system (Millipore, Bedford, MA, USA). All other regents were analytical grade. Sprague–Dawley rats (weighing 150–200 g, the same number of female and male) and Female Sprague–Dawley rats (about 250 g, pregnant for about 18 days) were purchased from the Hunan Academy of Traditional Chinese Medicine (Changsha, China).

Preparation and fractionation of PMs from hippocampus tissue

The rats were starved overnight and killed by decapitation. The PM purification procedures were carried out as described previously, with some modifications (Emmelot *et al*. 1974). Briefly, hippocampal tissues were excised and washed with ice-cold homogenization medium [0.25 M sucrose, 10 mM HEPES, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5] three times. All fresh tissues (about 2 g from 20 rats) were ground in a prechilled mortar and suspended in a four-fold volume of homogenization medium. The lysate was then centrifuged at 600 *g* at 4°C for 10 min. The pellet, including the nuclei and unbroken cells, was ground and centrifuged again. The combined supernatant was then centrifuged with 50 000 *g* at 4°C for 20 min. Some 3 mL 69% (w/w) sucrose solution was added drop by drop to the pellet with vigorous shaking in order to prevent membrane agglutination and subsequent contamination of the membranes. Each gradient, starting from the bottom of the tube, was composed of 2 mL each of 54, 45, 41 and 37% sucrose solution, and the homogenization medium was added to fill the tube. The sample was then centrifuged at 4°C at 70 000 *g* for 3 h. After centrifugation, fractions from each sucrose interface was collected and diluted 10 times with homogenization medium and then centrifuged for 30 min at 30 000 *g* at 4°C. Finally, a specimen of the pellet was analyzed by electron microscopy(Zhang *et al*. 2005). The other fractions were lysed directly in SDS sample buffer (4% SDS, 0.5 M Tris-HCl, 65 mM DTT, 20% glycerine) at room temperature (25°C) for 1 h with occasional vortexing. Then the sample was centrifuged at 13 000 *g* for 10 min at 4°C. The supernatant was stored at − 70°C until use.

Preparation of PMs from hippocampal primary neurons by affinity purification

Primary cultures of hippocampal neurons were prepared as described previously (Papa *et al*. 1995). Pregnant rats were anaesthetized with aether. The uteri were dissected in a dish filled with HBSS (144 mmol/L NaCl, 3 mmol/L KCl, 0.01 mmol/L Hepes, pH 7.5). Hippocampal tissue was rapidly dissected from fetal rat. The whole tissue was proteolytically digested at 37°C for 15 min, then washed with culture medium (76% Dulbecco's modified Eagle's medium supplemented with 10% F12, 10% fetal bovine serum and 150 mg/mL Gln) three times before rinsing and transferring it to culture bottles at 37°C. The next day the culture medium was substituted with growth medium (96% Neurobasal Medium (NB), 2% B27, 150 mg/mL Gln). On the fifth day, the culture medium was exchanged to a third medium (98% NB, 2% B27) and 10 mM FUDR was added (final concentration 0.1% in solution) to inhibit further glial proliferation. Thereafter, the culture medium was changed every 2 days. The primary neurons could be used for cell surface biotinylation after 10–12 days in culture.

Cell surface biotinylation was carried out essentially according to the manufacturer's instructions and relevant publications (Shin *et al*. 2003; Zhang *et al*. 2003; Peirce *et al*. 2004). Briefly, five dishes (10 cm diameter, about 10^7 cells/dish) were washed three times with prewarmed (37°C) phosphate-buffered saline (PBS), and then 3 mL PBS and 10 μL EZ-Link sulfo-NHS-SS-biotin stock solution (100 mg/mL, freshly prepared with dimethyl sulfoxide) were added. The reaction was carried out at room temperature for 10 min and unreacted sulfo-NHS-SS-biotin was quenched by the addition of Tris (pH 8.0) to a final concentration of 25 mM. After 10 min at room temperature, the cells were washed twice with PBS.

Biotinylated cells were scraped into ice-cold PBS and collected by centrifugation at 500 *g* for 5 min. The cells were resuspended in 10 mL ice-cold hypotonic buffer (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF) and incubated on ice for 15 min. The cells were disrupted by sonicating on ice using five 1-s bursts. Then the cells were incubated for 30 min on ice and vortexed for 5 s every 5 min. Unbroken cells and nuclei were pelleted from the cell homogenate by centrifugation at 800 *g* for 10 min at 4°C. The KCl concentration in the supernatant fraction was adjusted to 150 mM. A 300-μL aliquot of suspended streptavidin magnetic beads (prewashed with ice-cold hypotonic buffer four times before use) was added to the supernatant. The resulting suspension was rotated at 4°C for 1 h. The beads were collected using a magnetic plate. The affinity-purified membrane fraction was obtained after washing the beads three times with hypotonic buffer. The PM proteins were prepared by lysing in SDS sample buffer for 10 min at room temperature, and vortexing occasionally to insure complete lysis, and then centrifuged at 13 000 *g* for 10 min. The supernatant was collected, and the extraction was repeated once. The supernatants were combined for further analysis.

SDS–PAGE and western blotting analysis

PM proteins derived from affinity purification and density-gradient centrifugation were redissolved by adding a trace of bromophenol blue and heated at 100°C for 5 min. Samples were subjected to SDS–PAGE using an 11.5% separation gel and a 4.8% stacking gel. After electrophoresis, the gel was stained using Sypro Ruby. For western blotting analysis, the proteins were transferred to a PVDF membrane. The membrane was blocked with 5% non-fat dried milk in TBST (150 mM NaCl, 0.1% Tween-20, 25 mM Tris, pH 7.5) for 1 h at room temperature and then incubated with flotillin-1 monoclonal antibody (1 : 250 dilution) for 1 h with TBST containing 5% non-fat dried milk. After washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG for 1 h at room temperature. The membrane was washed with TBST again and the blot was developed using the Western Lightning Chemiluminescence Reagent.

In-solution and in-gel digestion of PM proteins

In-solution digestion of PM proteins from rat hippocampal tissue was performed as described previously with slight modification (Washburn *et al*. 2001). Briefly, 1 mg lyophilized protein was dissolved in 100 μL 70% trifluoroacetic acid and incubated for 5 min at room temperature. After adding 100 mg CNBr, the samples were incubated overnight at room temperature in the dark. On the following day, the pH was adjusted to 8.5 by the addition of ice-cold 25% $NH_3.H_2O$. Then solid urea was added to give a final urea concentration of 8 M. Protein disulfide bonds were reduced by adding DTT to a concentration of 1 mM for 50 min at 56°C, and carboxyamidomethylated in 10 mM iodoacetamide in the dark at room temperature for 30 min. The sample was then digested with endoproteinase Lys-C 1 : 100 (w/w) at 37°C overnight. After dilution to a urea concentration of 2 M with 25 mM NH_4HCO_3, the sample was digested once again with trypsin 1 : 50 (w/w) at 37°C overnight. The sample was centrifuged at 10 000 *g* for 10 min to

remove insoluble material and the supernatant transferred to a new microcentrifuge tube. The sample was concentrated with a SpeedVac to about 500 μL. After desalting by changing the buffer to aectonitrile (ACN) : H_2O (4 : 1) on a C18 reverse-phase (RP) column (4.6 mm × 250 mm, Vydac, Hesperia, CA, USA) using an HPLC instrument (Waters, MA, USA), the sample was lyophilized to dryness for MS analysis.

For one SDS–PAGE gel, the whole gel lane was cut into 50 slices and placed into clean Eppendorf tubes. In-gel digestion was done as decribed previously (Zhang *et al*. 2005). After digestion overnight, the gel pieces were extracted for 10 min in 100 μL 60% ACN containing 0.1% formic acid with ultrasonication. The supernatants were pooled and lyophilized in a SpeedVac to about 5 μL for MS analysis.

MS analysis and protein identification

The in-gel digested peptides were analyzed by electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) MS/MS. The settings and operations of this analytical procedure have been described in detail elsewhere (Zhang *et al*. 2005; Cao *et al*. 2006). The pkl files generated were analyzed using a licensed copy of the Mascot 2.0 program (MatrixScience Ltd, London, UK) on a 2.6-GHz Pentium-4 personal computer with 2 GB RAM, which compared the files against a rat protein non-redundant database (International Protein Index, IPI) containing 39 441 protein sequences (IPI_rat_v3.07) downloaded as fast-a formatted sequences from the European Bioinformatics Institute (EBI) (ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/). Search parameters and positive identification were set as described previously (Cao *et al*. 2006).

The in-solution digestion sample from hippocampal tissue was processed on a LTQ mass spectrometer (Thermo, San Jose, CA, USA) mainly according to Jiang *et al*. (2005). Briefly, RP-HPLC was performed using an Agilent 1100 Capillary System (Agilent Technologies, Wilmington, DE) on a C18 column (150 μm i.d., 100 mm long; Column technology Inc., Fremont, CA, USA). The pump flow rate was 1.6 μL/min. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in ACN. The tryptic peptide mixtures were eluted using a gradient of 2–55% B over 320 min. The acquired MS/MS spectra were also compared against the RAT IPI database (IPI_rat_v3.07) using the Turbo SEQUEST program in BioWorks 3.1 software (Thermo). All the out files were filtered by Buildsummary software to delete the redundant data. An accepted SEQUEST result must have a delta correlation (ΔCn) score of at least 0.1 (regardless of charge state). Peptides with a + 1 charge state were accepted if they were fully tryptic digested and had a cross-correlation (Xcorr) of at least 1.9. Peptides with a + 2 charge state were accepted if they had an Xcorr ≥ 2.2. Peptides with a + 3 charge state were accepted if they had an Xcorr ≥ 3.75. With these specific filter criteria, the remaining peptides showed some continuity to the *b* or *y* ion series, so we were fairly confident of the peptide identification with these strict filter criteria.

Data analysis and bioinformatics

A Perl script was written in house to parse significant hits from Mascot output files (html files) into tab delimited data files suitable for subsequent data analysis. An automated sequence retrieval script was written in Perl using the Bioperl libraries to generate FASTA formatted protein sequence from IPI databases for proteins identified by each MS experiment. The molecular mass values, pI values and percentage of the protein covered by the matched peptides were retrieved from Mascot output files. The average hydropathy for identified proteins was calculated using the ProtParam software (available at http://μsexpasy.org) by submitting each FASTA file in batch. The subcellular location and function of the identified proteins were elucidated by gene ontology (GO) component and function terms respectively, text-based annotation files of which were available for download from the GO database ftp site (ftp://ftp.geneontology.org/pub/go/) (Ashburner *et al*. 2000). The mapping of putative transmembrane (TM) domains in identified proteins was carried out using the transmembrane hidden Markov model (TMHMM) algorithm (available at http://www.cbs.dtu.dk/services/TMHMM) (Krogh *et al*. 2001).

Results and discussion

Isolation and characterization of PM from rat hippocampal tissue and primary neurons

Currently, there is no single proteome analysis strategy that can sufficiently address all levels of organization of the proteome. Proteomic analysis at the level of subcellular structures (that can be enriched by subcellular fractionation) is an analytical strategy that combines classic biochemical fractionation methods and tools for the comprehensive identification of proteins. Besides screening unknown gene products, this strategy may allow known but poorly characterized proteins to be identified. The efficiency of fractionation is critical for the information content of the whole study, such as the accuracy with which proteomics data allows one to assign potential newly discovered gene products to subcellular structures.

We used differential and density-gradient centrifugation to isolate the PM from freshly isolated rat hippocampal tissue (Fig. 1). Different fractions prepared by sucrose density-gradient ultracentrifugation were compared with whole-tissue lysate using an antibody against flotillin-1, a PM-specific protein (Nebl *et al*. 2002). As shown in Fig. 2(a), the fraction between the homogenization solution and 37% sucrose density was highly enriched in flotillin-1; the parts below the interface also contained some PMs, and were discarded in this analysis. Our results were consistent with previously published data obtained using a self-generating 35% percoll gradient to isolate brain PM; up to 10-fold enrichment of PM was observed in the top fractions of the gradient, which were substantially depleted of mitochondria and ER (Olsen *et al*. 2004; Nielsen *et al*. 2005). However, in a similar experiment to purify PM from rat liver (Emmelot *et al*. 1974), the PM gathered at the 37–41% sucrose interface. The reason for this discrepancy may be that the composition of biological membranes differs between tissues and their buoyant density depends on the content of protein, phospholipids and cholesterol. We also

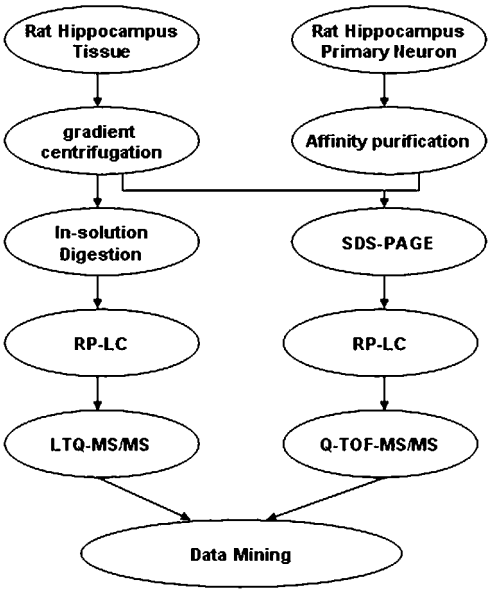

Fig. 1 Schematic representation of the strategy used to identify rat hippocampal PM proteins.

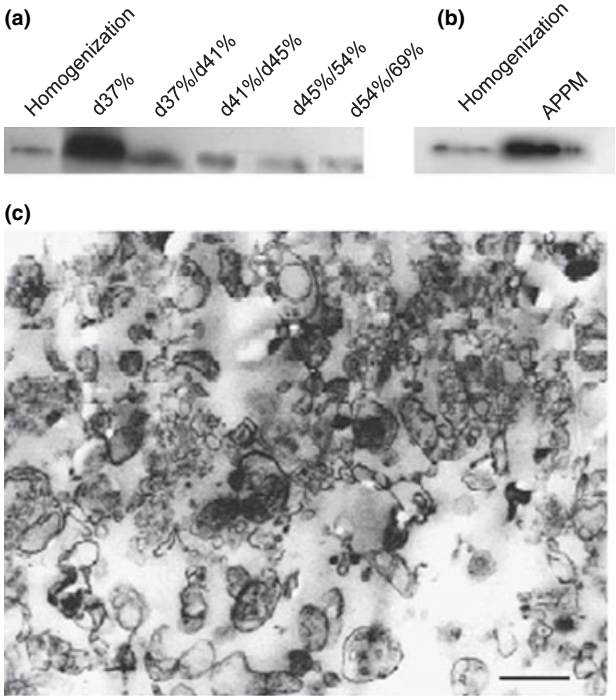

Fig. 2 Evaluation of PM purification strategies by western blotting and transmission electron microscopy (TEM). (a, b) Proteins (25 μg in each lane) were separated by SDS–PAGE (11.5% gel) and transferred to a PVDF membrane. The blots were probed with anti-flotillin-1 (48 kDa). (c) TEM analysis of the PM from hippocampal tissue. The bar represents 0.5 μm. Homogenization: whole-tissue lysate in (a), whole-cell lysate in (b); d37%, density above 37% on sucrose density gradient centrifugation; d37%/41%, density between 37 and 41%; d41%/45%, density between 41 and 45%; d45%/54%, density between 45 and 54%; d54%/69%, density between 54 and 69%. APPM, affinity-purified PM.

used electron microscopy to evaluate the purity of the fractions according to Stevens *et al*. (2003). As shown in Fig. 2(c), PMs were visible as interconnected black lines with membrane attached to their ends and sides. Very short pieces of membrane vesicles, which appeared as black dots, remained visible.

It is necessary to isolate a single cell type in order to characterize cell-specific proteins and to identify low-abundance proteins, such as ion channels, neurotransmitter receptors and other PM protein families. Laser-capture microdissection of brain has been used to isolate small groups of cells (Choudhary and Grant 2004), but there were artifacts induced by tissue staining and it was always equipment dependent. To obtain sufficient hippocampal neurons, we separated hippocampal cells from rat fetal brain by proteolytic digestion. Separated cells, still containing neuroglia, were cultured for 3–4 days, and then FUDR was added to block the proliferation of neuroglial cells, thus decreasing the possible glial contribution to negligible levels. After a further 7–8 days in culture, the primary cells, over 95% of which consisted of pyramidal-like, medium-sized neurons (10–20 μm) with many dendrites extending from the somata under phase-contrast microscopy (data not shown), were used for cell surface modification.

The biotin–avidin system and its application have been known for many years (Diamandis and Christopoulos 1991). Recently, it was applied to cell surface proteome research (Shin *et al*. 2003; Zhang *et al*. 2003; Peirce *et al*. 2004). For this purpose, many commercially or newly synthesized biotinylation reagents are available (Gauthier *et al*. 2004). In an effort to optimize biotinylation conditions, two reagents, sulfosuccinimidyl-6-(biotin-amido)hexanoate (sulfo-NHS-LC-biotin), recently used in several similar studies (Sabarth *et al*. 2002; Shin *et al*. 2003), and a more water-soluble analog, sulfo-NHS-SS-biotin, have been compared (Peirce *et al*. 2004; Scheurer *et al*. 2005). The results showed that sulfo-NHS-SS-biotin was superior to sulfo-NHS-LC-biotin not only in biotinylation specificity but also in efficiency (Peirce *et al*. 2004). Therefore, sulfo-NHS-SS-biotin was employed in this study. To isolate the PM, we used cell surface biotinylation with affinity enrichment by immobilized streptavidin beads (Fig. 1). Flotillin-1 was significantly enriched in the affinity-purified membrane fraction compared with whole-cell lysate (Fig. 2b).

Solubilization and separation of PM proteins

Solubilization of membrane proteins is a key issue in membrane biochemistry. For gel-based methods, to analyze the PM proteins from rat hippocampal tissue and primary neurons, 4% SDS sample buffer was used directly to solublize the purified PM proteins, which were then

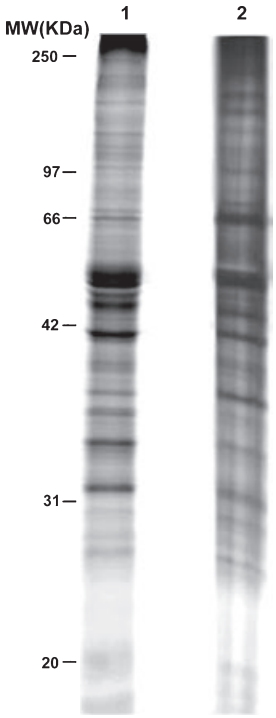

Fig. 3 SDS–PAGE analysis of purified PM protein. One hundred micrograms of protein was loaded and stained with Sypro Ruby. Lane 1, PM proteins purified from rat hippocampal tissue by sucrose density-gradient centrifugation; lane 2, PM proteins purified from rat primary neurons using a cell surface biotinylation and affinity purification strategy. MW, molecular weight.

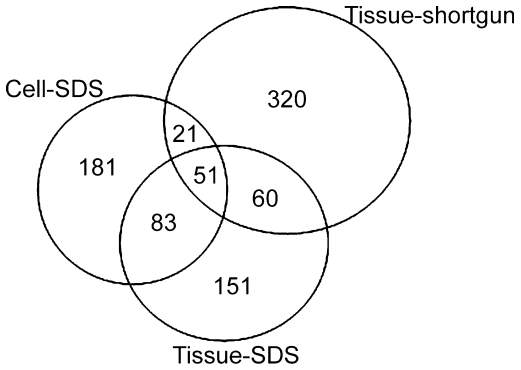

Fig. 4 Schematic diagram showing the overlap of proteins identified by different methods. Cell_SDS, proteins were purified from hippocampal primary neurons, separated by one-dimensional SDS–PAGE and identified by Q-TOF-MS/MS; Tissue_SDS, proteins were purified from hippocampal tissue, separated by one-dimensional SDS–PAGE and identified by Q-TOF-MS/MS; Tissue_Shotgun, proteins were purified from hippocampal tissue and identified by a shotgun strategy using LTQ-MS/MS.

separated by SDS–PAGE using 11.5% gels (Fig. 3). A high concentration of anionic detergent such as SDS can dissolve the sample efficiently, and little pellet was found in our centrifugation procedures. Shotgun methods provide a powerful alternative to gel-based methods. As with gel-based methods, the solubility of membrane proteins is also the major challenge for non-gel shotgun approaches. John R. Yates III's group provided the first large-scale proteomic analysis that included a substantial proportion of membrane proteins (Washburn *et al*. 2001). The method is robust and broadly applicable to various complex membrane samples. The shotgun strategy can be used as a complement to geLC-MS/MS, particularly for proteins with extreme molecular weight and a hydrophobic nature. In our analysis, an offline desalting and a long-duration RP were employed by the LC-LTQ-MS/MS.

Physicochemical characterization of the identified protein in rat hippocampal PM

The MS/MS spectra acquired were matched by searching the rat IPI protein sequence database. In order to avoid false-positive hits, first we used data only from the rat database to eliminate the low-confidence identification resulting from a non-specific database. Second, we referred to the parameters reported in previous studies and applied more strict criteria for peptide identification than those in most reported work (Peng *et al*. 2003; Jiang *et al*. 2005). Third, we manually checked the mass spectra of the identified peptides using different filters, which showed that the current criteria could give better quality MS/MS spectra. Using geLC-MS/MS methods with the Mascot algorithm, 345 and 336 non-redundant proteins were identified from rat hippocampal tissue and primary neurons respectively. The shotgun approach with the Sequest algorithm used to analyze the hippocampal tissue resulted in the identification of 452 non-redundant proteins in a single LC run, whereas integrating all the strategies enabled the identification of 867 unique proteins (supplementary Table S1), 687 from rat hippocampal tissue and 336 from primary neurons. Of these, 51 proteins were characterized in all three preparations and 181 proteins only in the primary neurons; 531 proteins were identified only in the tissue sample, with only 151 using the geLC-MS/MS and 320 using the shotgun strategy. The overlap proteins identified by the different strategies are shown in Fig. 4. Several physicochemical characteristics of the 867 identified proteins were defined, such as molecular mass, pI, hydrophobicity [grand average of hydropathy (GRAVY) value] and the number of TM domains (Fig. 5a–d).

In the present work, 556 (64.1%) of the 867 proteins, proteins are distributed in the range 10–60-kDa, compatible with general two-dimensional PAGE. The data in Fig. 5(a) indicate that the shotgun strategy was superior to the geLC-MS/MS strategy in the identification of proteins of high molecular weight. Regarding the pI distribution, a total of

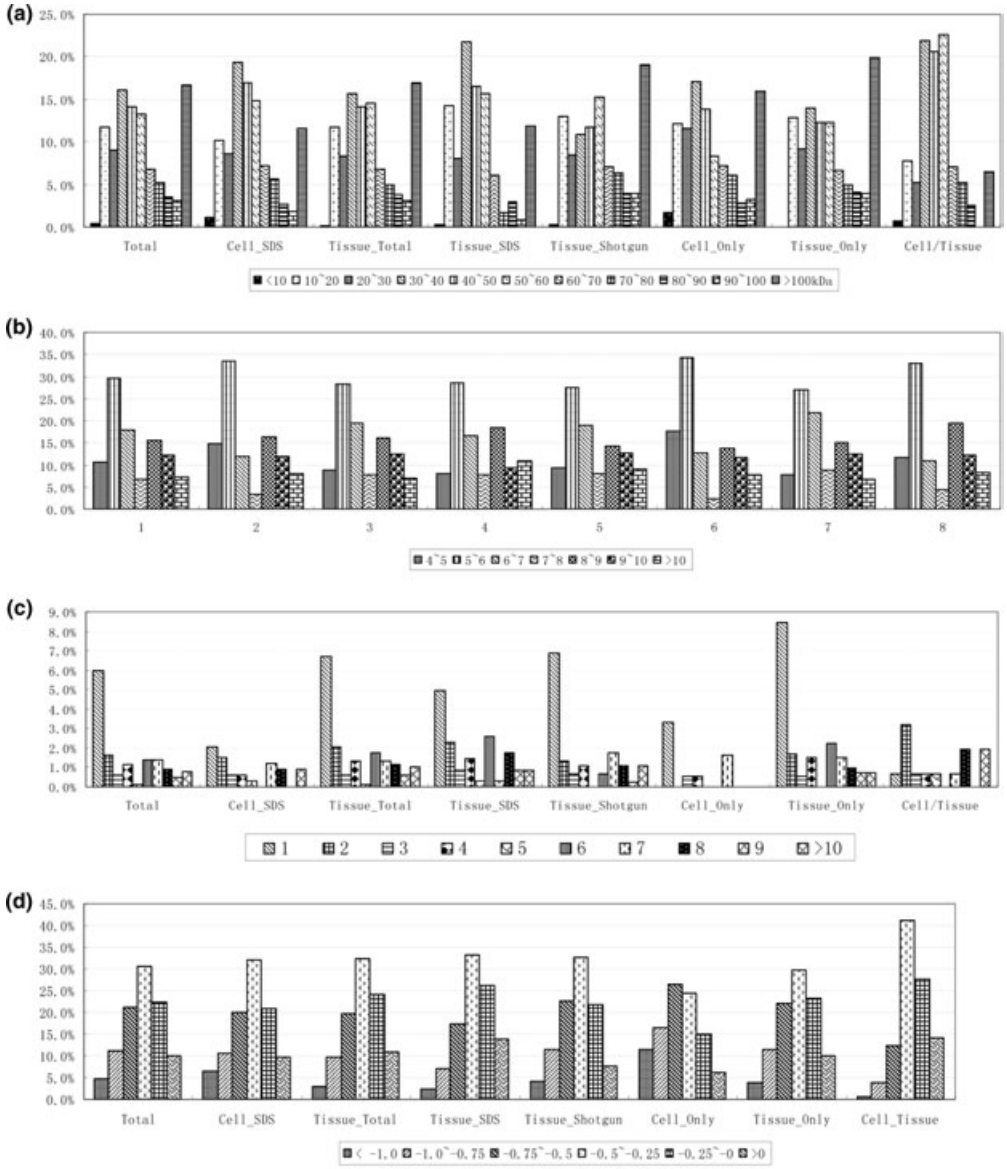

Fig. 5 Distribution of proteins identified from different fractions in relation to their theoretical mass (a), pI (b), GRAVY values (c) and the number of predicted helices (d). Cell_SDS, Tissue_SDS and Tissue_Shotgun are as indicated in Fig. 4. Tissue_all, proteins identified from rat hippocampal tissue; Tissue_Only or Cell_Only, proteins identified from hippocampal tissue only or primary neurons only; Cell/Tissue, proteins identified both in hippocampal tissue and primary neurons. The bars indicate the percentage of proteins in each fraction.

805 proteins (92.7%) are in the range pI 4–10 but four (0.5%) proteins have a pI < 4.3 and 63 proteins (7.3%) a pI > 10, also beyond the 2DE separation capability (Fig. 5b). When the calculated pI values were plotted against calculated molecular weights on a logarithmic scale (Fig. 6), we found that about 30% of these proteins fell outside the typical limits of protein resolution by 2DE (100 kDa; pI 4.5–8.5).

As regards the theoretical TM domains predicted by TMHMM, of the total 867 proteins, 125 (14.4%) have one or more predicted TM domain (Fig. 5c). Fifty-two (41.6%) of the membrane-spanning proteins contain only a single TM domain. Integral membrane proteins that are more hydrophobic, especially those with multiple TM domains, have fewer tryptic cleavage sites (hydrophilic residues R and K) and are frequently glycosylated, which may prevent access of the protease and reduce the number of possible identifiable tryptic peptides. Furthermore, some proteins lacking putative α-helices can be predicted to be anchored to the membrane as a result of post-translational modification, such as myristoylation and the prenylation. The hydrophobicity property of proteins is frequently expressed as the GRAVY index. For the 867 proteins we identified GRAVY values ranging from −1.684 to 0.816 (Fig. 5d). Only 86 proteins (9.9%) have

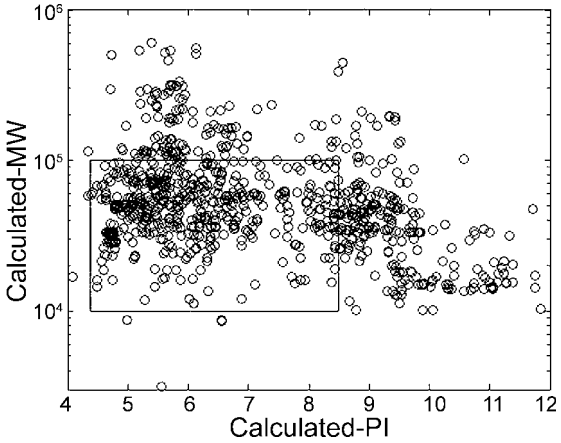

Fig. 6 Calculated pI of identified proteins plotted against calculated molecular weight (MW) on a logarithmic scale. Proteins not included in the box were beyond the typical limitations of 2DE.

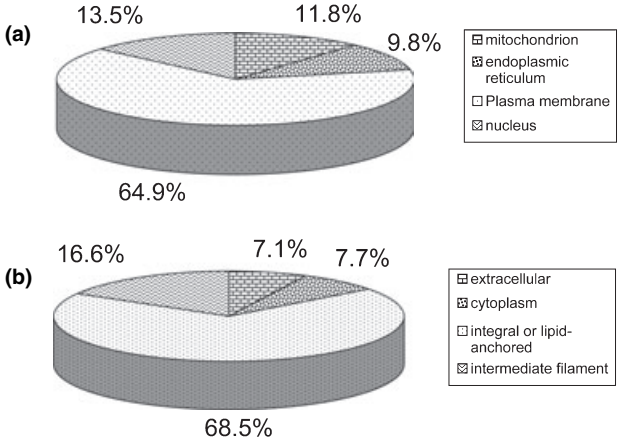

Fig. 7 Subcellular location of the identified proteins. (a) Expected primary subcellular localization of the characterized proteins. (b) Subclasses of PM proteins.

positive values. The majority of the analyzed proteins (55.8%) have a GRAVY value between + 0.4 and – 0.4, which could not discriminate their hydrophobic or hydrophilic nature (Molloy *et al*. 1998; Santoni *et al*. 2000). For instance, the Na^+/K^+-ATPase α–4 subunit (IPI00390795), which was characterized by all our strategies, and has 10 TM regions and is generally considered to be a highly hydrophobic protein, has a GRAVY value of only 0.034. On the other hand, ADP/ATP translocase 2 (IPI00366141), which does not have a predicted TM domain, has a value of 0.225.

Our data for the PM proteome showed slightly fewer TM proteins than several published reports (Zhao *et al*. 2004; Cutillas *et al.* 2005). This might be a consequence of our aim of investigating complete protein expression of the rat hippocampal PM, including integral membrane proteins as well as membrane-associated proteins; harsh washes with high-salt or high pH buffers were therefore avoided. In addition, the proteins isolated by the biotinylation and affinity purification approach are mainly cell surface proteins, including few TM proteins (Jang and Hanash 2003; Shin *et al*. 2003; Peirce *et al*. 2004), and not every protein on the cell surface will be extracted by this strategy owing to steric hindrance, lack of primary amines or a minimal sequence with extracellular exposure. In the primary neuron sample, only 27 proteins (8.0%) have a TM domain. However, 114 proteins (16.6%) were identified in the tissue sample with at least one TM domain (Fig. 5c).

Subcellular location and functional survey of hippocampal PM proteins

Eukaryotic cells segregate and organize specific molecules that carry out defined functions in organelles. The identification and delineation all of these molecules will provide the data necessary to understand organelle function. The subcellular locations of the identified proteins were categorized according to the universal GO cellular component annotation (Fig. 7a). Some 519 proteins (59.9%) have a GO annotation for a cellular component or cellular location, of which 337 (64.9%) could be associated with the PM. The remainder are localized primarily to ER (9.8%), mitochondrion (11.8%) and the nucleus (13.5%). These groups may include proteins that exist at more than one site in the cell. The data indicate that the contamination by mitochondria and ER in our membrane fraction was greatly reduced using our PM purification method.

We also classified the identified proteins according to their interaction with the PM as well as their orientation and structure in the membrane. Of the proteins residing at the PM (Fig. 7b), 231 (68.5%) are integral or lipid-anchored membrane proteins, 56 (16.6%) are cytoskeletal proteins, and 50 (14.8%) are externally or peripherally associated with the PM. The possible contamination of subcellular proteins may be due to other organelles in close contact with the PM, or the existence of proteins at more than one site in the cell. It is not unusual for proteins initially categorized as organelle specific to be discovered elsewhere in the cell at a later date. Several proteins identified here are examples of this. Elongation factor-Iα is known primarily as a component of the protein synthesis machinery, but has a less well known function in cytoskeletal reorganization (Negrutskii and El'skaya 1998). Protein disulfide isomerase, commonly classified as an ER protein, has recently been detected at the PM of platelets and liver (Honscha *et al*. 1993). Glucose-regulated-protein (GRP)78 (Bip) is an ER chaperone whose function is generally thought to be limited to the structural maturation of nascent polypeptides. It helps to prevent protein-folding intermediates from aggregating and to stabilize energetically unfavorable conformations of polypeptides to minimize irreversible protein misfolding (Dill and Chan 1997). However, in a previous study it was shown to be

expressed on the surface of several cancer cells (Shin *et al*. 2003). Other examples are histones, which are able to cross cell PMs and mediate penetration of macromolecules covalently attached to them (Khan *et al*. 1998; Hariton-Gazal *et al*. 2003). In our study, the histones were categorized in the nucleus according to the GO cellular component annotation, which may be one of the main reasons why we detected so many nuclear proteins (14.6%). It is often difficult to conclude whether these 'contaminants' represent true endogenous partners or artificial associations induced by cell disruption or incomplete purification. More recently, a comparative proteomics strategy was applied to analyze the relative levels of proteins in different organelle-enriched fractions, which can solve the problem of contaminants and distinguish between proteins from different subcellular compartments without the need to obtain pure organelles (Jiang *et al*. 2005). It seems a useful approach and could be used in our analysis to confirm the genuine PM proteins.

We also categorized the GO function annotations of the identified proteins (Fig. 8). Of 727 (83.9%) identified proteins with a GO function description, 31.8% and 24.6% are binding and catalytic proteins respectively; 14.1% are involved in cell structure molecular activity, and 6.8% have transporter activity that can mediate the movement of small molecules (ions and water) across the membrane bilayer. This group of proteins is known to be especially enriched in membranes. Other annotated proteins (6.6%) have special activities such as signal transducer activity, motor activity, antioxidant activity and so on.

The hippocampal PM proteome identified here was also classified into several functional groups according to the literature. The hippocampus plays an important role in memory storage and retrieval, and has an extensive ability to exchange information with other parts of the brain and sensory organs. Of the proteins identified in hippocampal PM (Table S1), several have been linked to signal regulation and neural transmission. For example, the protein soluble NSF attachment protein (SNAP)-25 (IPI00231420, IPI00204644) is highly expressed in neurons of the neocortex, hippocampus, piriform cortex, anterior thalamic nuclei, pontine nuclei, and granule cells of the cerebellum (Rizo and Sudhof 2002). Its role in disease development has not yet been clarified, but the data indicate that it is important. Specifically, it has been implicated in schizophrenia, in which the molecular pathology involves presynaptic components giving rise to abnormal exocytosis indicated by reduced amounts of SNAP-25 in hippocampus and increased amounts in CSF (Thompson *et al*. 2003). Changes in SNAP-25 expression levels represent early markers of synaptic loss (Ramirez *et al*. 2004). The levels of this protein were found to decrease in Alzheimer's disease and Down's syndrome (Greber-Platzer *et al*. 2003). Neuronal transmission requires a balance between synaptic vesicle exocytosis and endocytosis, while membrane fusion begins with the binding of vesicles to the target membrane via receptors. The PM proteins syntaxin (IPI00324381, IPI00191730) and SNAP-25 interact with high affinity and in an equimolar stoichiometry to form a stable dimer in the pathway to the ternary SNAP receptor (SNARE) complex (Rickman *et al*. 2004), which is an essential part in the process of exocytosis. Synaptotagmin-4 (IPI00231989) belongs to the synaptotagmin family, which are synaptic vesicle membrane proteins found in abundance in nerve cells and some endocrine cells. They have a regulatory role in membrane interactions during trafficking of synaptic vesicles at the active zone of the synapse. Neurotransmitter release and some other brain functions, such as short- and long-term modulation of synaptic efficacy, are regulated by calcium ions. Twenty-seven calcium-related proteins were identified from our data (Table 1). Calcium functions are mediated by calcium-binding proteins, including a group of proteins known as neuronal calcium sensor proteins. Calcium-dependent membrane association reversibly localizes neuronal calcium sensor proteins to distinct cellular signaling compartments and may mediate a critical mechanism for the coordinated regulation of the signaling cascade (Spilker *et al*. 2002).

Sorting of certain membrane proteins requires a mechanism involving rafts, protein–lipid complexes enriched in glycosphingolipids and cholesterol. These microdomains remain at the PM of different cell types and also play a significant role in signal transduction. In a proteomic analysis of the detergent-insoluble raft fractions from primary cultures of hippocampal neurons Ledesma *et al*. (2003) used a cholesterol removal strategy to confirm the specificity of the raft proteins. Several neuron-specific raft proteins were identified in their research, many of which were also included in our protein lists, such as heat-shock 70-kDa protein (IPI00368720, IPI00371863, IPI00480781), tubulin α1 (IPI00189795), tubulin α2 (IPI00339167), tubulin α6 (IPI00357912, IPI00364046), tubulin β5 (IPI00197579), and the known raft marker Thy-1 membrane glycoprotein (IPI00188956). Flotillin-1, another raft marker, which was used to evaluate the purity of our PM fractions, was not detected in our MS analysis, which may be due to the sensitivity of the this method.

Several transporters, pumps and channels were identified in the hydrophobic PM fraction. Glutamate is the excitatory

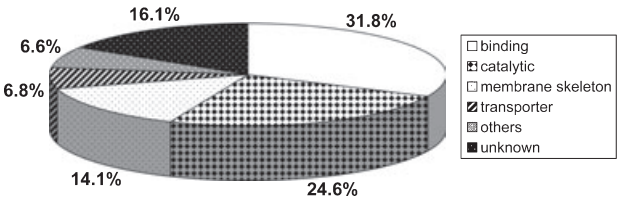

Fig. 8 Functional characterization of all GO-annotated proteins.

Table 1 All ion channels or related proteins

ID[a]	Protein description[b]	MW[c]	pI[d]	GRAVY[e]	TM[f]	Subcellular[g]	Function[h]	Workflow
IPI00194320	Sodium channel-associated protein 1A	80622	5.98	− 0.935	0	unchar	transporter	2
IPI00198327	Voltage-dependent anion-selective channel protein 2	32353	7.44	− 0.221	0	PM	transporter	2
IPI00198908	Chloride channel 2	54238.85	9.4	0.382	8	PM	transporter	3
IPI00199560	Chloride channel protein 2	99328.57	9.01	0.129	11	PM	transporter	3
IPI00207891	Splice isoform 1 of voltage-dependent anion-selective channel protein 3	31178	8.91	− 0.296	0	PM	transporter	1, 2
IPI00211872	Voltage-dependent l-type calcium channel β-1 subunit	65679.74	6.2	− 0.693	0	PM	transporter	3
IPI00211876	Voltage-dependent l-type calcium channel β-3 subunit	54564.19	5.85	− 0.746	0	ER	transporter	3
IPI00231067	Splice isoform 2 of voltage-dependent anion-selective channel protein 3	31309	8.91	− 0.289	0	PM	transporter	1, 2
IPI00231319	Voltage-gated potassium channel β-3 subunit	43689.71	8.53	− 0.288	0	PM	transporter	3
IPI00362007	Similar to voltage-dependent anion-selective channel protein 1 (VDAC-1) (mVDAC1) (mVDA)	34289	8.61	− 0.382	0	Mit	transporter	1, 2
IPI00365653	Similar to voltage-gated calcium channel α(2)δ-4 subunit	99046.2	5.7	− 0.248	0	PM	unknown	3
IPI00365813	Similar to voltage-dependent anion-selective channel protein 1 (VDAC-1) (mVDAC1) (mVDA	34114	8.5	− 0.387	0	Mit	transporter	1, 3, 2
IPI00368260	Similar to voltage-gated potassium channel β-1 subunit (K + channel β-1 subunit) (Kv-β-1)	10068.29	9.89	− 0.851	0	unchar	unknown	3
IPI00370826	Similar to voltage-dependent anion channel 1	22309	8.35	− 0.301	0	Mit	transporter	1, 2
IPI00372763	Calcium channel β4 subunit	57964.14	9.34	− 0.692	0	unchar	transporter	3
IPI00421662	Splice isoform 1 of voltage-dependent l-type calcium channel β-2 subunit	73226.52	8.49	− 0.9	0	ER	transporter	3
IPI00421663	Splice isoform 2 of voltage-dependent l-type calcium channel β-2 subunit	68175.24	8.88	− 0.907	0	ER	transporter	3
IPI00421664	Splice isoform 3 of voltage-dependent l-type calcium channel β-2 subunit	70988.05	8.83	− 0.942	0	ER	transporter	3
IPI00421665	Splice isoform 4 of voltage-dependent l-type calcium channel β-2 subunit	68951.15	8.8	− 0.908	0	ER	transporter	3
IPI00421666	Splice isoform 5 of voltage-dependent l-type calcium channel β-2 subunit	68065.93	8.34	− 0.904	0	ER	transporter	3
IPI00421874	Voltage-dependent anion-selective channel protein 1	32513	8.62	− 0.357	0	Mit	transporter	1, 2
IPI00192337	Calcium/calmodulin-dependent protein kinase type II α chain	54651	6.61	− 0.386	0	PM	catalytic	1, 3, 2
IPI00194873	Splice isoform D of PM calcium-transporting ATPase 1	138719.18	5.71	− 0.169	7	PM	catalytic	3
IPI00211185	Calcium/calmodulin-dependent protein kinase type II β chain	61105	6.73	− 0.353	0	PM	catalytic	2
IPI00212226	Splice isoform δ1 of calcium/calmodulin-dependent protein kinase type II δ chain	60080.63	6.84	− 0.424	0	unchar	catalytic	3, 2
IPI00213583	Splice isoform δ 6 of calcium/calmodulin-dependent protein kinase type II δ chain	57825.06	6.69	− 0.417	0	unchar	catalytic	3, 2
IPI00231267	Splice isoform A of PM calcium-transporting ATPase 1	129510.03	5.85	− 0.107	7	PM	catalytic	3
IPI00231268	Splice isoform B of PM calcium-transporting ATPase 1	136278.62	5.64	− 0.138	7	PM	catalytic	3
IPI00231269	Splice isoform C of PM calcium-transporting ATPase 1	137843.44	5.72	− 0.1	7	PM	catalytic	3

Table 1 Continued

ID[a]	Protein description[b]	MW[c]	pI[d]	GRAVY[e]	TM[f]	Subcellular[g]	Function[h]	Workflow
IPI00231270	Splice isoform E of PM calcium-transporting ATPase 1	129075.51	5.85	– 0.111	7	PM	catalytic	3
IPI00231271	Splice isoform K of PM calcium-transporting ATPase 1	130624.96	5.66	– 0.187	6	PM	catalytic	3
IPI00231612	Splice isoform δ2 of calcium/calmodulin-dependent protein kinase type II δ chain	56446.6	6.81	– 0.396	0	unchar	catalytic	3, 2
IPI00231613	Splice isoform δ3 of calcium/calmodulin-dependent protein kinase type II δ chain	57709.14	7.61	– 0.4	0	unchar	catalytic	3, 2
IPI00231614	Splice Isoform δ4 of calcium/calmodulin-dependent protein kinase type II δ chain	58549	7.01	– 0.396	0	unchar	catalytic	3, 2
IPI00326171	Calcium/calmodulin-dependent protein kinase II, β3 isoform	65847	6.85	– 0.346	0	PM	catalytic	2
IPI00358163	Similar to calcium-binding mitochondrial carrier protein Aralar2 (solute carrier famil)	102232	8.12	– 0.161	0	PM	binding	2
IPI00480684	Splice isoform δ5 of calcium/calmodulin-dependent protein kinase type II δ chain	54191.03	6.66	– 0.387	0	unchar	catalytic	3, 2
IPI00480796	Splice Isoform δ7 of calcium/calmodulin-dependent protein kinase type II δ chain	56293.43	6.82	– 0.386	0	unchar	catalytic	3, 2
IPI00205693	Sodium/potassium-transporting ATPase α-2 chain precursor	113457	5.39	– 0.007	8	PM	transporter	1, 3, 2
IPI00231451	Sodium/potassium-transporting ATPase α-3 chain	113045	5.26	– 0.007	8	PM	transporter	1, 3, 2
IPI00326305	Sodium/potassium-transporting ATPase α-1 chain precursor	114293	5.3	0.002	10	PM	transporter	1, 3, 2
IPI00339124	Sodium/potassium-transporting ATPase α-1 chain	35653	8.83	– 0.538	1	PM	catalytic	1, 2
IPI00188119	Splice isoform long of potassium-transporting ATPase α chain 2	115643	6.16	0.034	8	PM	transporter	2
IPI00231462	Splice isoform short of potassium-transporting ATPase α chain 2	103238	5.88	0.181	8	PM	catalytic	2

[a]IPI database accession number. [b]Description of the protein in IPI database. [c]Calculated molecular weight. [d]Calculated isoelectric point. [e]Value calculated using ProtParam algorithm. [f]Value predicted by TMHMM. [g]Primary subcellular location predicted according to the universal GO cellular component annotation. Mit, mitochondria; unchar, uncharacterized. [i]Protein identification strategies: 1, proteins were purified from hippocampal primary neurons, separated by one-dimensional SDS–PAGE and identified by Q-TOF-MS/MS; 2, proteins were purified from hippocampal tissue, separated by one-dimensional SDS–PAGE and identified by Q-TOF-MS/MS; 3, proteins were purified from hippocampal tissue and identified by a shotgun strategy using LTQ-MS/MS.

neurotransmitter to main classes of receptors, including intropic α-amino-3-hydroxy-5-methylisoxazole-4-propionate, kainate and NMDA receptors. In this study, we were able to identify four members of this group of proteins (IPI00231061, IPI00324625, IPI00389891, IPI00454545). Other excitatory amino acid transporters (IPI00213663, IPI00230956, IPI00231885, IPI00324377) were also in our hippocampal PM proteome. Disturbances in excitatory amino acid transporters have been suggested to contribute to the pathophysiology of hippocampus (Seal and Amara 1999). All the ion channel proteins or related proteins are listed in Table 1. Many of them are associated with brain disease (Hatta *et al*. 2002).

Most of the enzymatic proteins found in the PM fraction are involved in primary or secondary metabolism and only a few of these have TM domains. Lipids and their metabolism may be important for hippocampal development and function, as indicated by the number of identified lipid-related proteins. For example, apolipoprotein E (ApoE; IPI00190701) plays an important role in lipid transport in human blood and other body fluids. It participates in plasma lipoprotein metabolism, cholesterol homeostasis and local lipid transport processes (Mahley 1988). In the brain, it is primarily synthesized and secreted by astrocytes, and plays a major role in lipid transport within the CNS. Occurrence of the ApoE4 isoform is significantly associated with late-onset alzheimer's disease, but its exact role in the pathogenesis of Alzheimer's disease is not clear. ApoE may be involved in the formation of amyloid plaques or tangles by interacting with β-amyloid or tau proteins (Utermann 1994).

Its expression is considered to be a risk factor for Alzheimer's disease but is not necessarily sufficient for development of disease.

A number of structural proteins were also identified, including membranes of the intermediate filament, microfilament and microtubule, which cross-link with PM. The neurofilaments NF-L (IPI00231302) and NF-M (IPI00325609) are involved in the maintenance of neuronal caliber and stability. Fropomyosin (IPI00393158) is implicated in stabilizing cytoskeletal actin filaments, whereas ezrin (IPI00470254) connects to the PM via actin filaments. The complexity of the function of these proteins and the possible link between them and other identified proteins (such as phosphatases and kinases) suggest that these proteins may have a more extensive and important role in hippocampal function, possibly participating in information storage and memory.

Database analyses revealed that 140 (16.1%) of the proteins identified in this work are unknown proteins with no match with proteins of known function in other organisms. Many of them have not been described in the mammalian system, and even more have never been reported as hippocampal proteins. These proteins detected in the hippocampus are therefore valuable by-products and form the basis for challenging and innovative further research. A simple way to predict the function of these proteins is to consider their constituent domains. Sequence search against the Pfam database (Bateman *et al*. 2002) (http://www.sanger.ac.uk/software/pfam/index) showed that 27 uncharacterized proteins have one or more TM regions. Many of them have putative domains often found in PM proteins, such as Stomatin Prohibitin Flotillin HflKlC (SPFH) domains (Pfam: PF01145) B cell receptor-associated proteins (IPI00370387, IPI00361771, IPI00361973) and Cache domains (Pfam: PF02734) in proteins similar to voltage-gated calcium channel α-2 subunit and δ-4 subunit (IPI00365653). They can be envisaged as candidates for hippocampus-specific proteins and tentative markers, particularly when found in hippocampus-related diseases.

In our study, some disease-related or important differential proteins have been identified through search against our developed Differentially Expressed Protein Database (DEPD) database (He *et al*. 2005). For example, dihydropyrimidinase-related protein-2 (IPI00192034) and malate dehydrogenase (IPI00197696) have been reported to be related to morphine dependence (Kim *et al*. 2005). Dihydropyrimidinase-related protein-2 was detected only in morphine-treated cells of frontal cortex from rat brain, while the latter was under-expressed with morphine treatment. Other interesting proteins found include tubulin α-1 chain (IPI00189795), tropomyosin α-4 chain (IPI00214905) and some hypothetical proteins involved in neural stem cell differentiation and its Cyclin-Dependent-Kinases (CDK) pathway (Gillardon *et al*. 2005; Maurer *et al*. 2005).

Characterization of potential neuron-specific PM proteins

Hippocampal tissue is highly complex and heterogeneous, mainly including nerve neurons and neuroglial cells. Neurons can receive and respond to chemical or physical signals. They differ from other cells in having the ability to convert signals into electrical impulses using special ion channels. These impulses are conducted over long distances and transmitted to the next cell, be it another nerve cell, muscle or gland. Neuroglia, the 'other brain cells', constitute half of the weight of the brain but, being much smaller than neurons, they outnumber the neurons more than 10 times.

When protein levels are determined in neuronal cells or brain tissue, it is of great importance that the data be normalized with respect to a housekeeping protein and a protein marker for neuronal density to correct for neuronal loss. Surprisingly, few neuron-specific structures or neuroglial markers have been identified in rat or human brain by the proteomic approach so far (Lubec *et al*. 2003), and no systematic cell-specific PM studies have been reported. To reduce the complexity of the rat hippocampal proteome, and to increase the dynamic range and specificity for proteome detection, elementary PM proteomes of rat hippocampal tissue and primary neurons were described. In our analysis, 687 proteins from rat hippocampal tissue and 336 from primary neurons were identified (Fig. 4). Some 181 proteins were detected only in primary neurons, which can be considered as potential neuron-specific PM markers. At the same time, 531 proteins were identified only in the tissue sample, which could be regarded to some extent as potential neuroglia-specific proteins.

In this paper, we established an elementary database for rat hippocampal neuronal and neuroglia-specific PM proteins. Some reported single cell-specific proteins were characterized in our analysis. For example, neurofilament triple m protein, a reported neuron-specific protein, which is capable of assembly into the cytoskeleton and of forming a filamentous network, was identified only in our primary neuron fractions, which may indicate that it has low abundance in hippocampal tissue. Voltage-dependent anion-selective channel protein 1, also a neuron-specific protein expressed in PM and in mitochondria (Buettner *et al*. 2000; Bahamonde *et al*. 2003), was detected in both the tissue sample and the neuron sample (Lubec *et al*. 2003). γ-Enolase, which was localized in synaptic PM as an αγ heterodimer in rat brain (Ueta *et al*. 2004), is a neuron-specific enolase, which has neurotrophic and neuroprotective properties on a broad spectrum of central nervous system neurons. However, it was only characterized in the tissue fractions using the shotgun strategy, possibly because of the sensitivity of the different MS methods. Compared with neuronal proteins, research on glial markers, especially PM markers, is in its infancy and few proteins are reliable candidates. In a recent study, however, glial fibrillary acidic protein, a typical glial marker, was found in another cell type

as well (Bicknese *et al*. 2002). In our analysis, we detected this protein in the primary neuron sample, but this might have resulted from contamination during primary cell culture, even though over 95% of cells cultured were neuronal cells. It should also be remembered that glial fibrillary acidic protein is a highly abundant protein, with many isoforms and post-translationally modified forms observed in brain (Lubec *et al*. 2003). We must pay attention to the possibility that the neuronal protein profile is different in culture and *in vivo*, and that neuronal morphology, protein expression and function are affected greatly by physical and molecular cues from the surrounding environment (although with more sophisticated culture methods protein expression patterns in native tissue can now be maintained in culture). Furthermore, proteins identified here represent only a small fraction of the PM proteins expressed by hippocampal tissue or neurons. So, in order to detect the truly cell-specific PM proteins, efforts must be made comprehensively to characterize the PM proteome and to gain a better understanding of the membrane dynamics. In addition, other validation methods, such as western blotting or tissue immunostaining, could be used to confirm their orientations. Towards this end, our characterization of neuron-specific proteins represents a step towards the identification of a set of promising cell-specific PM markers, whose location must be confirmed by other experimental strategies.

Conclusions

We have used both geLC-MS/MS and shotgun strategies to study the PM proteome from rat hippocampal tissue isolated by sucrose density centrifugation. In addition, PM proteins were purified from hippocampal primary neurons by biotinylation and affinity purification, and analyzed using geLC-MS/MS. Western blotting results showed that our samples were enriched in PM proteins. Some 867 non-redundant proteins were identified, including 336 from primary neurons and 687 from hippocampal tissue. Of these, 64.9% were integral membrane or membrane-associated proteins. One hundred and eighty-one proteins were characterized only from the primary neurons, and can be viewed as potential cell-specific proteins. Further studies on hippocampus, aimed at characterizing additional PM proteins and confirming hypothetical PM proteins and neuronal or even neuroglial-specific proteins, will strengthen our knowledge of the hippocampus and the causes of pathological conditions such as Parkinson's disease, Alzheimer's disease and schizophrenia, which are related to hippocampal dysfunction.

Acknowledgements

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Supplementary material

The following supplementary material is available for this paper online.

Table S1 All identified proteins

Table 2(a) All Q-TOF-MS/MS data

Table 2(b) All LTQ-MS/MS data

This material is available as part of the online article from http://www.blackwell-synergy.com

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