Proteomic Analysis of Brain Plasma Membranes Isolated by Affinity Two-phase Partitioning*[S]

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A comprehensive analysis of plasma membrane proteins is essential to in-depth understanding of brain development, function, and diseases. Proteomics offers the potential to perform such a comprehensive analysis, yet it requires efficient protocols for the purification of the plasma membrane compartment. Here, we present a novel and efficient protocol for the separation and enrichment of brain plasma membrane proteins. It lasts only 4 h and is easy to perform. It highly enriches plasma membrane proteins and can be applied to small amounts of brain tissue, such as the cerebellum of a single rat, which was used in the present study. The protocol is based on affinity partitioning of microsomes in an aqueous two-phase system. Marker enzyme assays demonstrated a more than 12-fold enrichment of plasma membranes and a strong reduction of other compartments, such as mitochondria and the endoplasmic reticulum. 506 different proteins were identified when the enriched proteins underwent LC-MS/MS analysis subsequent to protein separation by SDS-PAGE. Using gene ontology, 146 proteins were assigned to a subcellular compartment. Ninety-three of those (64%) were membrane proteins, and 49 (34%) were plasma membrane proteins. A combined literature and database search for all 506 identified proteins revealed subcellular information on 472 proteins, of which 197 (42%) were plasma membrane proteins. These comprised numerous transporters, channels, and neurotransmitter receptors, *e.g.* the inward rectifying potassium channel Kir7.1 and the cerebellum-specific γ-aminobutyric acid receptor GABRA6. Surface proteins involved in cell-cell contact and disease-related proteins were also identified. Six of the 146 assigned proteins were derived from mitochondrial membranes and 5 from membranes of the endoplasmic reticulum. Taken together, our protocol represents a simple, rapid, and reproducible tool for the proteomic characterization of brain plasma membranes. Because it conserves membrane structure and protein interactions, it is also suitable to enrich multimeric protein complexes from the plasma membrane for subsequent analysis. ***Molecular & Cellular Proteomics 5:390–400, 2006.***

Plasma membrane (PM)[1] proteins play pivotal roles in various physiological processes of the brain, such as signal transduction, molecular transport, and cell-cell interactions. PM proteins include neurotransmitter receptors, G-proteins, carriers, and voltage-gated ion channels. Many of them display region- and time-specific expression patterns, therewith determining network specificity and information processing. The characterization of PM proteins is, therefore, essential for a better understanding of brain structure and function. Proteomics has the potential to effectively profile PM proteins and to provide unprecedented insight into the protein composition of a given sample. However, profiling PM proteins has proven to be particularly challenging because of their low abundance and the difficulties in resolving and identifying them. To overcome these limitations, new technologies are constantly under development. They include liquid chromatography coupled to mass spectrometry (LC-MS), thus obviating two-dimensional gels that are not suitable for separation of membrane proteins (1, 2). They also involve novel strategies, such as protein tagging, which are aimed at a high enrichment of PM proteins (3, 4). Ultimately, the new technologies should be capable of assessing the dynamic nature of expression, interactions, and posttranslational modifications of PM proteins (5). Furthermore, proteomic analyses should be applicable to very small tissue samples, *e.g.* to functionally or anatomically defined brain areas from model organisms such as rats and mice (6). Toward this goal, here we introduce a novel protocol for the isolation of PMs from microsomes. It is based on affinity purification in an aqueous two-phase system consisting of the polymers polyethylene glycol (PEG) and dextran. When solutions of these two structurally different polymers are mixed, aqueous two-phase systems can form,

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[1] The abbreviations used are: PM, plasma membrane; PEG, polyethylene glycol; WGA, wheat germ agglutinin; MS/MS, tandem mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid; amu, atomic mass units; GPI, glycosylphosphatidylinositol; RSA, relative specific activity.

with the top phase being enriched in PEG and the bottom phase enriched in dextran (7). Membranes of different subcellular origin distribute in the aqueous two-phase system according to membrane surface properties such as charge and hydrophobicity. Consequently, they become enriched in either of the two phases. As PMs have a higher affinity to the PEG-enriched phase than all other membranes, they end up preferentially in the top phase (8). Further separation of PMs from contaminating membranes can be obtained by the subsequent use of the affinity ligand wheat germ agglutinin (WGA) coupled to dextran (9, 10). As *N*-acetyl-D-glucosamine and sialic acid are surface molecules of PMs but not of other microsomal membranes and because both molecules bind strongly to WGA, the selective pulling of PMs into the WGA-dextran-enriched phase is enabled. Since glycosylated and non-glycosylated PM proteins are co-distributed in the same membrane patches, both protein groups will co-partition in the WGA-dextran enriched bottom phase. In contrast, all other membranes remain in the PEG-enriched top phase. Furthermore, the aqueous two-phase system provides a mild environment preserving protein interactions. Hence, it can be combined with other procedures, such as blue native gel electrophoresis (11, 12), to characterize specific protein complexes. Our protocol omits time- and material-consuming density gradient centrifugation and yields PMs of high purity within 4 h. The suitability of the protocol was assessed by a SDS-PAGE coupled to LC-MS/MS of the PM proteome of the cerebellum of a single young adult rat, resulting in the identification of 506 proteins, of which 34–42% were PM proteins.

EXPERIMENTAL PROCEDURES

Tissue Preparation—Sprague-Dawley rats of both genders (8–9 weeks old) were deeply anesthetized by a peritoneal injection of 700 mg/kg chloral hydrate and sacrificed by decapitation. All protocols complied with the current German Animal Protection Law and were approved by the local animal care and use committee (Landesuntersuchungsamt, Koblenz, Germany). The brains were rapidly removed and stored at −80 °C until further usage. The cerebellum was dissected from freshly prepared brains in a chilled (~4 °C) solution containing 25 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 260 mM D-glucose, 2 mM sodium pyruvate, 3 mM *myo*-inositol and 1 mM kynurenic acid, pH 7.4. After dissection, the tissue was stored at −80 °C until further usage.

Chemicals—Dextran T500, Tris, Tween-20, glycine, and sucrose were purchased from Roth (Karlsruhe, Germany). Solvents and modifiers for liquid chromatography were obtained from Merck (Darmstadt, Germany). All other chemicals were from Sigma-Aldrich (Munich, Germany).

Preparation of Microsomes—Microsomes were obtained by differential centrifugation as described previously (13). During the establishing phase of the protocol, 1.5 g of brain tissue were homogenized in 4.5 ml of 250 mM sucrose, 15 mM Tris, pH 7.4, by 20 strokes with rotation (250 revolutions/min) in a glass Teflon homogenizer at 4 °C. When the protocol was ultimately assessed in combination with LC-MS/MS analysis, the cerebellum of a single rat was used. Nuclei were pelleted by 10 min of centrifugation at 3,000 × *g* in a table top centrifuge, and the mitochondrial pellet was obtained by 12 min of centrifugation at 10,000 × *g* in the same centrifuge. The supernatant was centrifuged for 1 h at 100,000 × *g* in a SW40 rotor to yield the microsomal pellet. The nuclear pellet and the mitochondrial pellet were re-extracted twice.

Preparation of WGA-Dextran—All solvents used for activation of dextran were dried using molecular sieves; all glass material was dried in an oven. Freeze-dried dextran was activated with tresyl chloride, again freeze-dried, and stored at −20 °C; WGA was coupled to tresyl dextran as described previously (14).

Aqueous Two-phase Affinity Partitioning—The two-phase affinity partitioning procedure is illustrated in Fig. 1. All steps were performed at 4 °C. Two-phase systems were prepared the day before use and stored in a refrigerator over night. The next day, 400 μl of resuspended microsomes were added to 3.6 g of a two-phase system, thus forming a 4 g two-phase system with microsomes (6.3% (w/w) PEG 3350, 6.3% (w/w) dextran T500, and 15 mM Tris/H_2SO_4, pH 7.8). The two-phase system was mixed by 20 invertations, vortexing, and another 20 invertations. Phase separation was accelerated by 5 min of centrifugation at 150 × *g*. After phase separation, top phase 1 was removed and stored until further usage. The bottom phase was re-extracted with an equal volume of a fresh top phase (obtained from a two-phase system without microsomes). After mixing and phase separation, top phase 2 was removed and combined with the stored top phase 1. The combined top phases 1 and 2 were then layered on a fresh bottom phase of a 4-g two-phase system. After mixing and phase separation, the resulting top phase 3 was removed and extracted with a fresh bottom phase of a 4-g system prepared as above. The resulting top phase 4 was removed and mixed with a fresh affinity bottom phase of an 8-g system (6.3% (w/w) PEG 3350, 6.3% (w/w) dextran T500, 800 μg of WGA in the form of WGA-dextran, 2 mM Li_2SO_4, 15 mM Tris borate, pH 7.8) to pull PMs into the dextran-enriched bottom phase. After mixing and phase separation, top phase 5 was discarded, and the bottom phase was washed with the same volume of a fresh top phase from a novel 8-g two-phase system (6.3% (w/w) PEG 3350, 6.3% (w/w) dextran T500, 2 mM Li_2SO_4, 15 mM Tris borate, pH 7.8). After mixing and phase separation, the resulting top phase 6 was discarded, and the bottom phase was diluted 10-fold with 100 mM *N*-acetyl-D-glucosamine, 250 mM sucrose, and 5 mM Tris, pH 7.8, to release the membranes from the WGA-dextran. The membranes were finally pelleted by centrifugation at 100,000 × *g* for 1.5 h in a SW40 rotor.

High Salt Washing and High pH Washing—To enrich integral PM proteins prior to LC-MS/MS analysis, the membranes obtained through two-phase affinity partitioning were resuspended in an ice-cold solution of 1 M KCl and 15 mM Tris, pH 7.4 (high salt washing). After 15 min on ice, the solution was centrifuged at 233,000 × *g* for 1 h in a SW40 rotor. The pellet was re-extracted twice with the high salt solution and subsequently washed three times with 0.1 M Na_2CO_3, pH 11.5 (high pH washing) as described (15). Both the high salt solution and the high pH solution reduce non-covalent protein-protein interactions, thus lowering the amount of peripheral proteins that were non-covalently bound to PM proteins (16). Moreover, the high pH solution causes depolymerization of actin bundles, thus resulting in their removal from the pellet (17).

Protein Assays—Protein amounts were determined using the method of Bradford (18) with bovine serum albumin as standard. Marker enzymes for the various cellular compartments were as follows: alkaline phosphatase (EC 3.1.3.1) was used as a PM marker (19), succinate dehydrogenase (EC 1.3.5.1) as a marker for mitochondria (19), and NADH ferricyanide reductase (EC 1.6.2.2) as a marker for the endoplasmic reticulum (20).

One-dimensional-PAGE of Purified Plasma Membrane Proteins—The affinity-purified, high salt, and high pH-treated proteins (30–50 μg) were resuspended via ultrasonication in 25 μl of lithium dodecylsulfate-sample buffer (Invitrogen). After incubation at 75 °C for 15

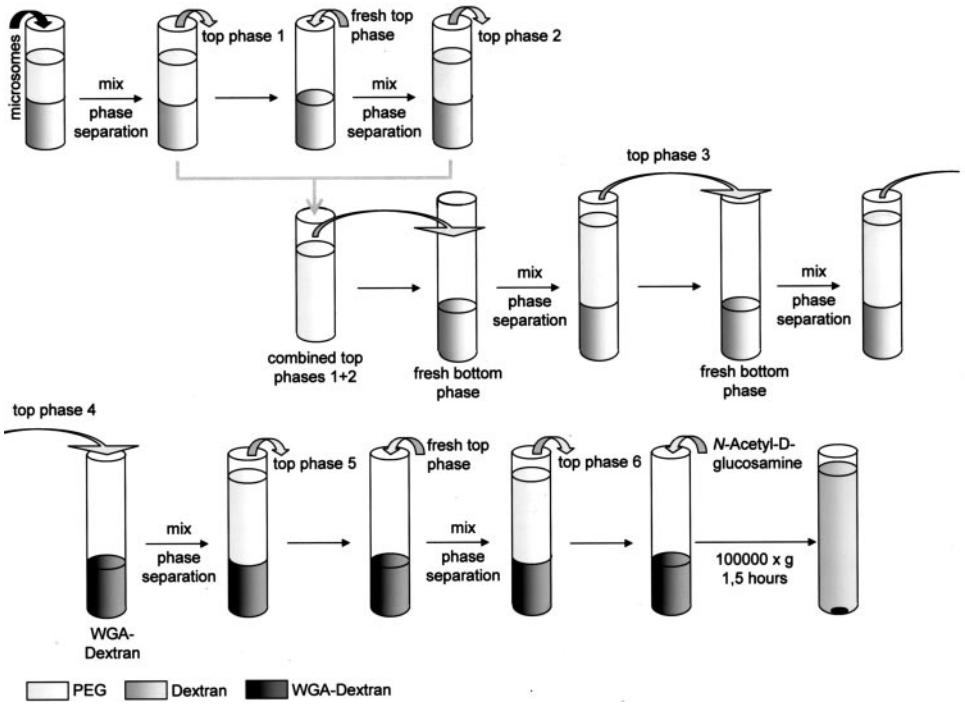

FIG. 1. **Affinity partitioning procedure for the enrichment of brain PMs.** The complete procedure is described under "Experimental Procedures." Microsomes obtained by differential centrifugation were separated in a two-phase system. PMs enriched in the top phase were collected, and the bottom phase was re-extracted (*first row*). The combined top phases enriched in PMs were washed twice by fresh bottom phases (*second row*). After washing, the top phase was applied to an affinity bottom phase coupled with WGA as affinity ligand for plasma membranes (*third row*). The affinity bottom phase (WGA-dextran), enriched in PMs, was washed with fresh top phase and diluted with *N*-acetyl-D-glucosamine to release the PMs, which were finally concentrated by sedimentation.

min, the sample was applied to a 4–12% Bis-Tris gel with a MOPS buffer system (NUPAGE®-Novex, Invitrogen). Protein separation occurred for 15 min at 50 V and an additional 60 min at 200 V at 4 °C. Visualization of protein bands was performed by a modified colloidal Coomassie staining (21). Briefly, the gel was immersed for 16 h in a solution containing 34% (v/v) methanol, 2% (v/v) phosphoric acid (89%), 17% (w/v) ammonium sulfate, and 0.066% (w/v) Coomassie G250. Background gel staining was reduced by gentle washing in water. Subsequently, the gel lanes were cut in 1-mm slices and frozen at −80 °C until further usage.

Sample Processing for Mass Spectrometry—Proteins were in-gel reduced and carbamidomethylated prior to tryptic digestion. Each gel slice was alternately washed three times with 50 mM ammonium hydrogen carbonate and 25 mM ammonium hydrogen carbonate, 50% acetonitrile, followed by incubation at 57 °C for 30 min with 10 mM dithiothreitol. Upon cooling to room temperature, the proteins were carbamidomethylated with 10 mM iodoacetamide for 30 min followed by two alternate washing steps as stated before. The gel pieces were dried, and proteins were subjected to trypsin proteolysis by adding 5 μl of a 25 μg/ml trypsin solution (sequencing grade, Promega, Madison, WI) in 50 mM ammonium hydrogen carbonate and incubating at 37 °C for 16 h. Peptides were extracted by incubation in 15 μl of 0.1% trifluoroacetic acid for 30 min at 37 °C.

Mass Spectrometry—Separation of tryptic peptide mixtures was achieved by nano-scale reversed-phase liquid chromatography in combination with online electrospray ionization (ESI)-MS. For the HPLC separation, a nano-LC system (Famos™, Switchos™, Ultimate™, Dionex, Idstein, Germany) was used, employing a linear gradient with a slope of 0.5 or 1% B/min (solvent A: 0.1% formic acid; solvent B: 0.1% formic acid, 84% acetonitrile) to a final concentration of 50% B. Followed by a 5-min washing period with 95% B, the columns were equilibrated in 5% B prior to the application of the next sample. The column system consisted of a precolumn (300 μm inner diameter × 1 mm length, C18 PepMap™) and a separation column (75 μm inner diameter × 150 mm length, C18 PepMap™), both purchased from Dionex. Mass spectrometric analysis was performed on a Qtrap4000 linear ion trap system (Applied Biosystems, Darmstadt, Germany). For online coupling, a micro ion spray source (Applied Biosystems) was used, equipped with a New Objective ESI needle (10 μm tip diameter, MS Wil, Wil, Switzerland). Typical values for needle voltage were 2.1–2.4 kV in positive ion mode. The declustering potential was set to 60 V, and the collision gas was set to high. A typical scan cycle consisted of an enhanced multiple charge survey scan (mass range 415–1,500 amu; 4,000 amu/s scan rate) followed by an enhanced resolution scan (250 amu/s scan rate) of the three most intense signals in the spectrum with an exclusion list for ion signals set to 30 s after one occurrence. Enhanced product ion spectra were recorded within the linear ion trap for doubly and triply charged precursor ions (mass range 100–1,500 amu; 4,000 amu/s scan rate), enabling the identification of major immonium ions as well as sufficient ion series information for successful processing via database search algorithms. Data acquisition was accomplished using the Analyst 1.4 software (Applied Biosystems).

Mass Spectrometric Data Interpretation—The derived mass spectrometric datasets were converted to Mascot Generic Format and searched against the Swiss-Prot database (release 46.0) with the species subset *Rattus* using the Mascot™ search algorithm (Matrix Science, London, UK). Generation of peak lists was accomplished using a Mascot script (mascot.dll, version 1.6b15, Matrix Science) as plug-in to the Analyst 1.4 software. All peaks below 0.1% of overall

intensity were removed; spectra were centroided and doubly as well as triply charged precursors with more than 50 peaks were searched against the Swiss-Prot database.

As for the search parameters, the number of allowed miscleavages was set to one, and carbamidomethylation was chosen as fixed, and oxidation of methionine was chosen as variable modification. Ion tolerance for peptide and fragment ions was set to 0.4 amu while the search was confined to doubly and triply charged precursors. Only unique ion scores were monitored while discarding all scores below 29 for individual peptide hits. A protein hit was usually based on at least two identified peptides with subsequent manual validation. In case of assignments based on single spectra, the peptide sequence, Mascot™ score, and the observed mass and charge were included in the supplementary material.

Bioinformatics—Gene ontology searches were performed using the EASE program (apps1.niaid.nih.gov/david/) (22). Myristoylated proteins (23), glycosylphosphatidylinositol (GPI)-anchored proteins (24) as well as proteins containing hydrophobic α-helical domains (23) were predicted using the sequence analysis tools of the Institute of Molecular Pathology, Vienna, Austria (mendel.imp.univie.ac.at/mendeljsp/index.jsp). A manual search for subcellular location was based on literature and Genecards (bioinformatics.weizmann.ac.il/cards/index.shtml).

RESULTS

Establishment of Affinity Two-phase Partitioning on Brain Tissue—As an alternative to density gradient centrifugation, PMs can be isolated by affinity partitioning of microsomes in aqueous two-phase systems (9, 25). To establish an affinity partition system for brain PMs obtained from small amounts of tissue, we started with a protocol reported for the extraction of rat liver PMs (9). Microsomes were prepared by differential centrifugation and partitioned in a PEG/dextran two-phase system using WGA-dextran as affinity ligand. Unfortunately, subsequent enzyme marker assays revealed a strong contamination by mitochondria, which we identified by succinate dehydrogenase activity (data not shown). Furthermore, it was unclear whether the protocol resulted in an optimal enrichment of brain PMs. Therefore we modified the original protocol in several aspects. Because the partitioning of subcellular compartments between the phases strongly depends on polymer and salt concentrations (7), several modifications of the concentrations of these components were performed to assess whether fractionation could be improved. In a first step, various concentrations of dextran T500 and PEG 3350 were explored, ranging from 5.4% to 6.9% (w/w), thus deviating from the value of 5.7% (w/w) used in the original protocol applied for rat liver PMs. At a concentration of 6.3% (w/w), PMs became most efficiently enriched in the top phase (Fig. 2). Furthermore, they became separated from the mitochondria, which comprised the main contamination. In a second step, the effect of different concentrations of Li_2SO_4 on the WGA-dextran affinity partitioning was analyzed. Li_2SO_4 influences membrane partitioning in the two-phase systems by generating a potential across the two phases (7). The bottom phase, which accumulates SO_4^{2-}, becomes more negative than the top phase, which contains most of the Li^+. The resulting potential moves the negatively charged membranes into the top phase. Separation of PMs from other membranes in the bottom phase was improved compared with the original protocol (which used no Li_2SO_4) when 2 mM Li_2SO_4 was applied. Additionally, to further increase the yield and purity of the PMs, we adjusted the amount of WGA. Without WGA, only 23.5% of the PM marker alkaline phosphatase and 40.9% of the protein were recovered in the bottom phase (Fig. 2). Increasing amounts of WGA coupled to dextran led to increased recovery in the bottom phase. Highest recovery was obtained using 100-μg WGA/1 g system, which resulted in 84.9% of the PM marker activity and 59.4% of total protein amount in the bottom phase (data not shown).

Finally, we modified the washing procedure. Instead of applying the combined top phases 1 and 2 directly to the WGA-dextran bottom phase, as it was done in the original protocol, we inserted several re-extraction and washing steps in between (Fig. 1).

Enzyme Marker Assays—To evaluate the final protocol, enzyme assays were performed. To preserve enzyme activity, the PMs were not exposed to high salt and high pH washing. The marker enzyme alkaline phosphatase was used as a PM marker, whereas the mitochondrial succinate dehydrogenase and the NADH ferricyanide reductase of the endoplasmic reticulum were employed as markers of the two major contaminating compartments (Table I). 10.4% of alkaline phosphatase activity was recovered in the microsomes compared with the activity obtained from the starting material. Determination of the relative specific activity (RSA) revealed a 2.4-fold enrichment. After affinity partitioning, 5.3% of the initial alkaline phosphatase activity was recovered in the final pellet, and the RSA value demonstrated a 12.3-fold enrichment compared with the starting material. Thus, the differential centrifugation for preparation of microsomes resulted in a loss of almost 90% of the PM marker and in an only 2.4-fold enrichment, whereas the affinity partitioning procedure (*cf.* Fig. 1) resulted in a recovery of 51% and an additional 5.1-fold enrichment (Table I, compare yield and RSA between microsomes and pellet). This demonstrates the high efficiency of the affinity partitioning procedure. As demonstrated by succinate dehydrogenase activity, mitochondria were removed by 96.2% in the microsomes and by 99.8% after the affinity partitioning procedure. Thus, the contamination by mitochondria was further reduced 19-fold by applying the affinity partitioning procedure. The combination of differential centrifugation and affinity partitioning resulted in a final RSA of 0.5 compared with 0.8 obtained with microsomes, showing that mitochondria are further removed from PMs by the affinity partitioning procedure. NADH ferricyanide reductase activity revealed that the endoplasmic reticulum was removed by 94% in the microsomes and by 99.6% after the affinity partitioning procedure. Again, this shows the potential of our protocol to reduce contaminating compartments from the PMs. RSA values of 1.3 (microsomes) and 0.8 (final pellet) also show the benefits of the affinity partitioning procedure for the

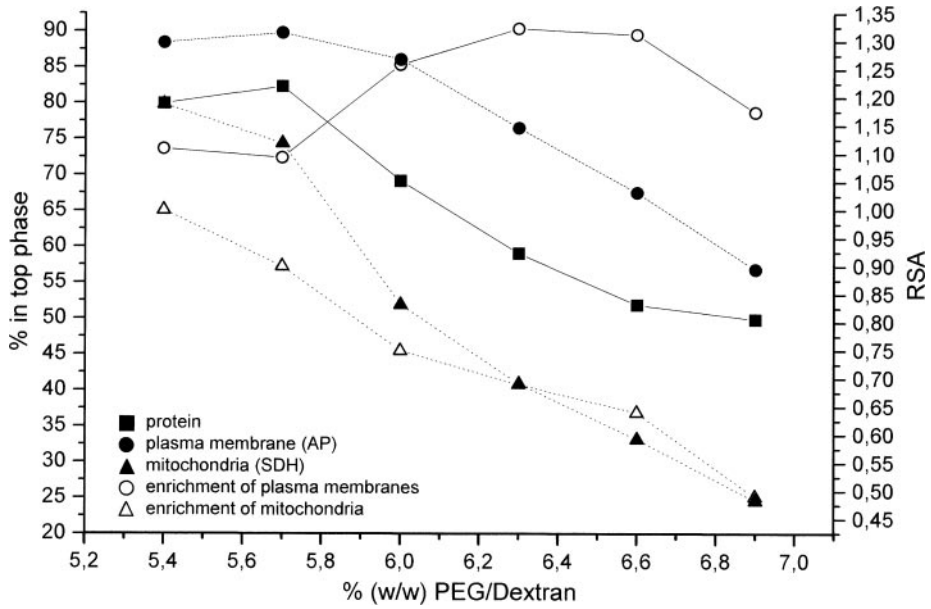

FIG. 2. **Partitioning of total proteins, PMs, and mitochondria in two-phase systems with various polymer concentrations.** The effect of various polymer concentrations was assessed by marker enzyme assays. Alkaline phosphatase (*AP*) activity and succinate dehydrogenase (*SDH*) activity were used as PM marker and mitochondrial marker, respectively. Note that the percentage of PM marker activity in the top phase declined at polymer concentrations > 6.0% (w/w), indicating that high polymer concentrations have negative effects on the recovery in the top phase. However, the RSA of the PM marker (*i.e.* the ratio between PM marker activity and protein amount) reached the highest value at a concentration of 6.3% (w/w) PEG/dextran, demonstrating that these polymer conditions lead to the highest enrichment of PMs in the top phase. Also note that the RSA of the mitochondrial marker SDH was substantially reduced in the top phase at a polymer concentration of 6.3% (w/w). Although an even lower value was obtained at a polymer concentration of 6.9% (w/w), we chose to use the 6.3% (w/w) condition for our subsequent analyses. Data represent mean values of three independent experiments.

TABLE I
Distribution of marker enzymes

Yield and relative specific activity (RSA) of alkaline phosphatase (EC 3.1.3.1) (PM marker), succinate dehydrogenase (EC 1.3.5.1) (mitochondrial marker), and NADH ferricyanide reductase (EC 1.6.2.2) (marker for endoplasmic reticulum) were determined as the average of three independent experiments ± standard deviation in homogenate, the microsomal pellet, and the final pellet obtained through the affinity partitioning procedure. The RSA values obtained for the mitochondrial and the endoplasmic reticulum marker were reduced from the homogenate to the final pellet (mitochondria: from 0.8 to 0.5, which is equivalent to a 1.6-fold reduction; endoplasmic reticulum: from 1.3 to 0.8, equivalent to a 1.6-fold reduction). In contrast, the RSA of the PM marker increased in the procedure (from 2.4 to 12.3, which is equivalent to a 5.1-fold enrichment).

	Protein	Plasma membrane		Mitochondria		Endoplasmic reticulum	
	Yield	Yield	RSA	Yield	RSA	Yield	RSA
	%	%		%		%	
Homogenate	100	100	1	100	1	100	1
Microsomes	4.8 ± 1	10.4 ± 1.7	2.4 ± 0.9	3.8 ± 1.2	0.8 ± 0.3	6 ± 0.5	1.3 ± 0.2
Final pellet	0.5 ± 0.1	5.3 ± 1.2	12.3 ± 3.4	0.2 ± 0.1	0.5 ± 0.4	0.4 ± 0.1	0.8 ± 0.3

removal of the endoplasmic reticulum. Taken together, the enzyme and Bradford assays imply a high yield of PMs with our approach because the PM marker activity in the final pellet amounted to 5.3% of the value obtained in the homogenate, whereas only 0.5% of the original protein amount was recovered. This conclusion is further supported by the low values of mitochondrial and endoplasmic reticulum marker activity (0.2% and 0.4%, respectively). Furthermore, the enrichment of PMs (RSA = 12.3) is paralleled by a reduction of contaminating compartments (mitochondrial RSA = 0.5; endoplasmic reticulum RSA = 0.8).

LC-MS/MS Analysis of Affinity-purified Plasma Membranes from Cerebellum—To determine the extent of contamination by a method independent of marker enzymes and to demonstrate the suitability of the protocol for small amounts of tissue, affinity-purified PM proteins from the cerebellum (weight about 300 mg) of a single rat were analyzed by SDS-PAGE coupled to LC-MS/MS. As integral PM proteins are of primary interest for most neuroproteomic approaches, the affinity-purified PMs were washed with high salt and high pH buffers to remove peripheral and vesicle-enclosed membrane proteins (15). Proteins that were insoluble under high salt and

TABLE II
Statistical analysis of identified proteins

Identified proteins from 50 μg protein separated by a short gradient (1% increase in solvent B per minute) and from 30 μg protein separated by a long gradient (0.5% increase in solvent B per minute) were investigated for their subcellular location using gene ontology and gene cards. Between 34 and 46% of all allocated proteins could be assigned to plasma membranes, depending on the sample and database used. Furthermore, at least one transmembrane helix was predicted for ~50% of the proteins.

	50 μg gel	30 μg gel	Both gels
Number of identified proteins	351	447	506
With subcellular location (gene ontology)	107	132	146
membrane	72	87	93
integral to membrane	56	68	74
plasma membrane	38	46	49
integral to plasma membrane	27	29	31
integral to mitochondrial membrane	6	5	6
integral to endoplasmic reticulum membrane	5	5	5
With subcellular location (gene cards)	317	416	472
plasma membrane	146	180	197
mitochondria	32	29	37
endoplasmic reticulum	17	23	24
With 1 or more predicted transmembrane helices	188	226	249
With more than 1 predicted transmembrane helices	126	154	168
With predicted GPI-anchor	10	8	10
With predicted myristoylation	12	14	15

high pH conditions were separated by 4–12% SDS-PAGE. To do so, two gels were loaded with 50 or 30 μg of protein. Gel slices of equal size (about 1 mm) were excised and subjected to in-gel digestion using trypsin. The resulting peptides were extracted from each gel slice and analyzed by nano-LC-MS/MS for protein identification with either a short gradient (1% increase in solvent B per minute with the 50-μg samples) or a long gradient (0.5% increase in solvent B per minute with the 30-μg samples). The independent analysis of the sample by slightly different protocols was performed to demonstrate the reproducibility of the datasets obtained. This resulted in the identification of 351 proteins for the 50-μg sample and 447 proteins for the 30-μg sample. The higher number of identified proteins from the 30-μg sample is due to the extended gradient providing improved peptide separation. Combining both gels, a total of 506 distinct proteins were identified (Tables II and supplemental Table I) with 182 proteins (38.9%) identified with a single peptide. Using gene ontology, 146 proteins (29%) were assigned to their subcellular location. Ninety-three (64%) of them were annotated as membrane proteins, of which 74 (51%) were integral membrane proteins. Forty-nine (34%) of the 146 proteins were PM proteins, with 31 (21%) being integral PM proteins (Table II). Six (4%) proteins were from mitochondrial membranes, and 5 (3%) were allocated to the membranes of the endoplasmic reticulum. Similar results were obtained when the datasets of the two gels were analyzed separately (Table II). Because only ~30% of the identified proteins could be allocated to their subcellular location when using gene ontology, we extended our analysis and performed a literature search to find further information about the subcellular location. This search resulted in the subcellular allocation of 472 proteins, of which 197 (42%) were PM proteins (supplemental Table I) with 55 proteins (27.9%) identified with a single peptide.

Because transmembrane domains, GPI anchor, and myristoylation consensus sequences are indicative of membrane proteins, the identified proteins were also analyzed for these motifs to provide an additional criterion for the evaluation (Table II, supplemental Table I). Among the 506 proteins identified, 249 (49.2%) proteins were predicted with at least one transmembrane domain and 168 (33.2%) with more than one transmembrane domain; 10 (2%) of the identified proteins were predicted to have a GPI anchoring site, and 15 (2.9%) were predicted to have a myristoylation site that could anchor these proteins in the PM. Taken together, the results confirm the high enrichment of PM proteins obtained with our affinity partitioning protocol.

Functional Categories of Identified Proteins—The identified proteins cover a broad range of different functions and belong to distinct families. Eighty-four of the classified proteins are listed in Table III. We identified several proteins involved in neurotransmission, *i.e.* proteins acting as neurotransmitter release machinery, postsynaptic receptors, or neurotransmitter re-uptake systems. Twelve proteins were identified which mediate transmitter release through the fusion of synaptic vesicles with the PM: 8 syntaxin proteins, namely STX1A, STX1B2, STX2, STX3, STX4, STX6, STX7, STX8; the syntaxin-associated protein unc18-homolog; and 3 synaptosomal associated proteins, namely SNAP23, SNAP25, and SNAP29. Among the neurotransmitter receptors, 14 distinct proteins were identified: the glutamate receptor subunits mGluR1, mGluR2, mGluR5, GluR1, GluR2, GluR3, GluR4, and GluRd2; the γ-aminobutyric acid receptors GABRA1, GABRA6, GABRB1, GABRB2 and GABRD; and the purinergic receptor

TABLE III

Selected plasma membrane proteins from cerebellum

Selected proteins that were identified by LC-MS/MS were categorized in neurotransmitter release machinery, neurotransmitter receptors, neurotransmitter re-uptake system, primary and secondary transporters and non-receptor type channels, cell-cell communication, and disease-related PM proteins. For each protein, the Swiss-Prot accession number, the protein name, the number of predicted transmembrane helices (TMH) (23), and the number of different identified peptides are indicated.

Accession no.	Protein name	TMH	Identified peptides
Neurotransmitter release			
P32851	Syntaxin-1A (STX1A)	1	8
P61265	Syntaxin-1B2 (STX1B2)	1	27
P50279	Epimorphin (STX2)	1	1
Q08849	Syntaxin-3 (STX3)	1	3
Q08850	Syntaxin-4 (STX4)	1	7
Q63635	Syntaxin-6 (STX6)	1	8
O70257	Syntaxin-7 (STX7)	1	7
Q9Z2Q7	Syntaxin-8 (STX8)	1	2
P61765	Syntaxin binding protein 1 (unc 18-homolog)	0	13
O70377	Synaptosomal-associated protein SNAP23	0	3
P60881	Synaptosomal-associated protein SNAP25	0	24
Q9Z2P6	Synaptosomal-associated protein SNAP29	0	2
Neurotransmitter receptors			
P62813	γ-aminobutyric acid receptor α-1 (GABRA1)	5	3
P30191	γ-aminobutyric acid receptor α-6 (GABRA6)	4	1
P15431	γ-aminobutyric acid receptor β-1 (GABRB1)	5	1
P63138	γ-aminobutyric acid receptor β-2 (GABRB2)	5	2
P18506	γ-aminobutyric acid receptor δ (GABRD)	5	1
Q9EPX4	P2Y purinoceptor 12 7 1	7	1
P23385	Metabotropic glutamate receptor 1 (mGluR1)	8	2
P31421	Metabotropic glutamate receptor 2 (mGluR2)	7	1
P31424	Metabotropic glutamate receptor 5 (mGluR5)	6	2
P19490	Glutamate receptor 1 (GluR1)	5	2
P19491	Glutamate receptor 2 (GluR2)	4	2
P19492	Glutamate receptor 3 (GluR3)	4	2
P19493	Glutamate receptor 4 (GluR4)	5	1
Q63226	Glutamate receptor delta-2 subunit (GluRd2)	4	8
Neurotransmitter re-uptake systems			
P24942	Excitatory amino acid transporter 1 (EAAT1)	10	18
P31596	Excitatory amino acid transporter 2 (EAAT2)	12	15
O35921	Excitatory amino acid transporter 4 (EAAT4)	8	10
P23978	Na^+/Cl^--dependent GABA transporter 1 (GAT1)	12	5
P31647	Na^+/Cl^--dependent GABA transporter 3 (GAT3)	12	9
P31646	Na^+/Cl^--dependent GABA transporter 2 (GAT2)	13	1
P28572	Na^+/Cl^--dependent glycine transporter 1 (GlyT1)	12	5
P31662	Orphan Na^+/Cl^--dependent neurotransmitter transporter NTT4	11	5
Primary and secondary transporters and non-receptor type channels			
P06685	Na^+/K^+-transporting ATPase α-1 chain (ATP1A1)	8	50
P06686	Na^+/K^+-transporting ATPase α-2 chain (ATP1A2)	9	65
P06687	Na^+/K^+-transporting ATPase α-3 chain (ATP1A3)	8	56
P07340	Na^+/K^+-transporting ATPase β-1 chain (ATP1B1)	1	19
P13638	Na^+/K^+-transporting ATPase β-2 chain (ATP1B2)	1	11
Q63377	Na^+/K^+-transporting ATPase β-3 chain (ATP1B3)	1	5
P11505	Plasma membrane Ca^{2+}-transporting ATPase 1 (ATP2B1)	10	14
P11506	Plasma membrane Ca^{2+}-transporting ATPase 2 (ATP2B2)	9	38
Q64568	Plasma membrane Ca^{2+}-transporting ATPase 3 (ATP2B3)	11	25
Q64542	Plasma membrane Ca^{2+}-transporting ATPase 4 (ATP2B4)	10	3
P23562	Band 3 anion transport protein (AE1)	15	9
P23347	Anion exchange protein 2	10	1
Q01728	Na^+/Ca^{2+} exchange protein NCX1	11	3
P48768	Na^+/Ca^{2+} exchange protein NCX2	12	5
Q63633	K^+/Cl^--cotransporter 2 (KCC2)	14	18
P55016	$Na^+/K^+/Cl^-$-cotransporter NKCC2	12	1
P11167	Facilitated glucose transporter 1 (Slc2a1)	11	3
Q07647	Facilitated glucose transporter 3 (Slc2a3)	11	2

TABLE III—*continued*

Accession no.	Protein name	TMH	Identified peptides
P43427	Facilitated glucose transporter 5 (Slc2a5)	12	1
Q9JJZ1	Facilitated glucose transporter 8 (Slc2a8)	12	1
P53987	Monocarboxylate transporter 1 (MCT1)	11	4
P29975	Aquaporin 1	7	4
P47863	Aquaporin 4	6	9
O70617	Inward rectifier potassium channel 13 (Kir7.1)	2	4
P49655	Inward rectifier potassium channel 10 (Kir4.1)	2	1
P08050	Gap junction alpha-1 protein (Cx43)	4	12
P54900	Na^+-channel β-2	2	2
P04775	Na^+-channel protein type II α	24	1
P10499	Potassium voltage gated channel Kv1.1	6	3
Q01956	Potassium voltage gated channel Kv3.3	7	2
Q63881	Potassium voltage gated channel Kv4.2	7	1
P54290	Dihydropyridine-sensitive Ca^{2+} channel α-2/δ	3	1
Q9Z2L0	Voltage-dependent anion-selective channel 1	0	12
Cell-cell communication			
Q63604	BDNF/NT-3 growth factor receptor	2	2
Q63198	Contactin 1	2	51
P22063	Contactin 2	1	9
P97846	Contactin associated protein 1	3	4
Q9Z0J8	Neuronal growth regulator 1 (kilon protein)	1	10
P13596	Neural cell adhesion molecule 1 (N-CAM140)	2	28
P97686	Neuronal cell adhesion molecule (Nr-CAM)	2	10
P97685	Neurofascin	3	23
Q62765	Neuroligin	3	1
Q07310	Neurexin 3-α	2	1
Q62718	Neurotrimin	2	7
Disease-related PM proteins			
P08592	Amyloid β A4 protein	2	4
P13852	Major prion protein	3	3
Q8CGU6	Nicastrin	2	4
Q9JK11	Nogo (Reticulon 4)	3	9
P61805	Defender against cell death 1	3	3
O88407	Fas apoptotic inhibitory molecule 2	7	2

P2Y12. Within the category of neurotransmitter re-uptake systems, several types of transporters were found: 3 excitatory amino acid transporters (EAAT1, EAAT2, and EAAT4); 3 γ-aminobutyric acid transporters (GAT1, GAT2 and GAT3), the glycine transporter GlyT1, and the orphan Na^+/Cl^--dependent neurotransmitter transporter NTT4. Another important class of neuronal PM proteins contains primary transporters, secondary transporters, and non-receptor type channels, of which 33 were identified. The primary transporter proteins included 6 subunits of the Na^+/K^+-ATPase (ATP1A1, ATP1A2, ATP1A3, ATP1B1, ATP1B2, and ATP1B3) and 4 Ca^{2+}-ATPase subunits (ATP2B1, ATP2B2, ATP2B3, and ATP2B4). The secondary transporter proteins comprised the Cl^-/HCO_3^- exchanger AE1, the anion exchange protein 2, the Na^+/Ca^{2+} exchangers NCX1 and NCX2, the K^+/Cl^--cotransporter KCC2, the $Na^+/K^+/Cl^-$-cotransporter NKCC2, the facilitated glucose transporters Slc2a1, Slc2a3, Slc2a5, Slc2a8, and the monocarboxylate transporter MCT1. Twelve nonreceptor type channel proteins were identified: the water channels aquaporin 1 and aquaporin 4, the inward rectifying potassium channels Kir7.1 and Kir4.1, the gap junction protein Cx43, the sodium channel beta-2 subunit, the alpha subunit of sodium channel type II, potassium voltage gated channels Kv1.1, Kv3.3, and Kv4.3, the dihydropyridine-sensitive Ca^{2+}-channel alpha2/delta, and the voltage-dependent anion-selective channel protein 1. Another class represented surface proteins for cell-cell communication. Within this class, we identified 11 proteins: the BDNF/NT3 growth factor receptor, contactin 1 and 2, the contactin-associated protein 1, the kilon protein, N-CAM 140, Nr-CAM, neurofascin, neuroligin 1, neurexin 3, and neurotrimin. Finally, six disease-related PM proteins were identified: the amyloid beta A4 protein, the major prion protein, nicastrin, Nogo, and two apoptosis-related proteins, namely defender against cell death 1 and Fas apoptotic inhibitory molecule 2. Aside from PM proteins, our analysis also revealed contaminating proteins of various origins (supplemental Table I). A great number of these could be categorized into 6 groups. The first group comprised mitochondrial proteins (37 of 472), *e.g.* cytochrome C oxidase subunit 2, phosphate carrier protein, ATP synthase protein 8, and monoamine oxidase. The second group was composed of proteins of the endoplasmic reticulum. Examples are the

sarcoplasmic/endoplasmic reticulum calcium ATPases 1 and 2, calnexin, and the protein disulfide-isomerase A3. The third group consisted of synaptic vesicle membrane proteins, of which we identified several Rab proteins, synaptotagmins, synaptobrevins, synaptophysin, the synaptic vesicle protein 2, the cysteine string protein, and the vesicular inhibitory amino acid transporter VIAAT. Proteins of the translation machinery composed the fourth group and consisted of 36 ribosomal proteins and the elongation factor EF1a1. The fifth and sixth group comprised cytoskeleton proteins (20 proteins) and chaperons (6 proteins), respectively. In summary, the protocol introduced in the present study is able to allocate as much as 34–42% of the identified proteins to a PM origin. It is thus feasible to enrich PMs from minute brain samples and make them available for proteomic analysis.

DISCUSSION

This study presents a novel fractionation protocol for neural PM proteins that is based on affinity two-phase partitioning. Fractionation by affinity partitioning is simple and requires no specific equipment. It is selective and much more rapid than conventional membrane preparation protocols. The highly specific enrichment of PMs by this approach is illustrated by marker enzyme analysis, which demonstrated a 12.3-fold enrichment compared with the initial homogenate. This enrichment is in good agreement with the 34–42% PM proteins, identified by gene ontology or literature search, as they constitute between 0.4–2.5% of the homogenate (26). The use of a single enzyme activity as criterion for yield and enrichment might lead to erroneous estimations because of the heterogeneity in the partitioned membrane patches. An alternative might therefore be the immunoblot analysis against multiple marker proteins. We used enzyme activity measurements because these demonstrate a broader linear range compared with immunoblot analysis and because enzyme tests were used in several previous analyses of two-phase systems (9, 10, 25). Furthermore, the subsequent identification of pre- and postsynaptic PM proteins by mass spectrometry argues against a preferential enrichment of different membrane components.

An important aspect in brain proteomics is the ability to analyze anatomically or functionally defined areas. Conventional protocols for the isolation of PMs from tissue combine two techniques, differential centrifugation and subsequent density gradient centrifugation. Most of these protocols are time- and material-consuming, and the yield of highly purified PMs is often low (14). Partitioning in an aqueous two-phase system, in contrast, requires only small amounts of material. The purification of PMs from fat cells by differential and density-gradient centrifugation required 16–24 rats, whereas partitioning in an aqueous two-phase system required only 1–2 rats (29). This efficacy is confirmed by our data, which demonstrate that less than 300 mg of tissue are sufficient as starting material. Thus, small brain structures, such as the cerebellum of a single rat, can be analyzed. The protocol probably can further be downscaled by circumventing the initial preparation of microsomes. Being based on differential centrifugation, this step led to a loss of 90% of the initial PM protein amount and yielded only a 2.4-fold enrichment, whereas the affinity partitioning itself led to a 5-fold enrichment of PMs with a recovery of 50%. Other improvements may be the replacement of the SDS-PAGE coupled to LC-MS/MS analysis by multidimensional liquid chromatography coupled to mass spectrometry (1, 30).

Therefore our protocol compares favorably with previously reported protocols of PM purification from brain tissue (27, 28). The most important criteria for assessing the quality of protocols are the enrichment and yield of PM proteins. Whereas PM proteins constituted only 12–15% in the studies by Nielsen *et al.* (27) and 20% in the study by Olsen *et al.* (28), our protocol yielded 34–42% PM proteins. This represents a 2- to 3.5-fold higher enrichment of PM proteins as compared with the previously reported protocols. With respect to the starting material, all three studies applied similar amounts of tissue, *i.e.* between 150–300 mg. Only when using a much more sensitive and accurate FT-ICR mass spectrometry, Nielsen *et al.* (27) succeeded in the analysis of as little as 15–20 mg brain tissue and identified 1,685 different proteins. This high number of different proteins, as compared with our study, is likely due to the fact that they used the FT-ICR mass spectrometry, different criteria for data validation, and a different database for protein annotation. Furthermore, they used mouse tissue, where more proteins are represented in the database than for rat. In the Swiss-Prot database, for instance, there are currently 9893 entries for mouse protein entries and 4544 for rat proteins. Therefore, the number of identified PM proteins depends on several factors and is hardly comparably between the different approaches used in the three studies. Another advantageous feature of affinity partitioning is that the aqueous polymer environment is gentle to membrane structure and function and preserves protein interactions (7). Aqueous polymer two-phase systems were used for binding studies and analysis of protein interactions (8, 31, 32). The protocol is therefore suitable to enrich multiprotein complexes of the PM, which are a major focus in proteome analysis (33–36). After affinity partitioning, these signaling complexes can be isolated further by complex-specific affinity techniques, such as immunoprecipitation or blue native gel techniques (11, 12). In contrast, the high salt, high pH, and urea treatment in the stepwise depletion protocol used by Nielsen *et al.* (27) likely destroys protein complexes. Taken together, our protocol provides the highest enrichment of PM proteins from small amount of brain tissue by simultaneously preserving protein complexes.

To validate our novel protocol, we analyzed the PM proteome of the rat cerebellum and identified many proteins known to be important for function of this brain region. These included proteins involved in neurotransmission, such as the

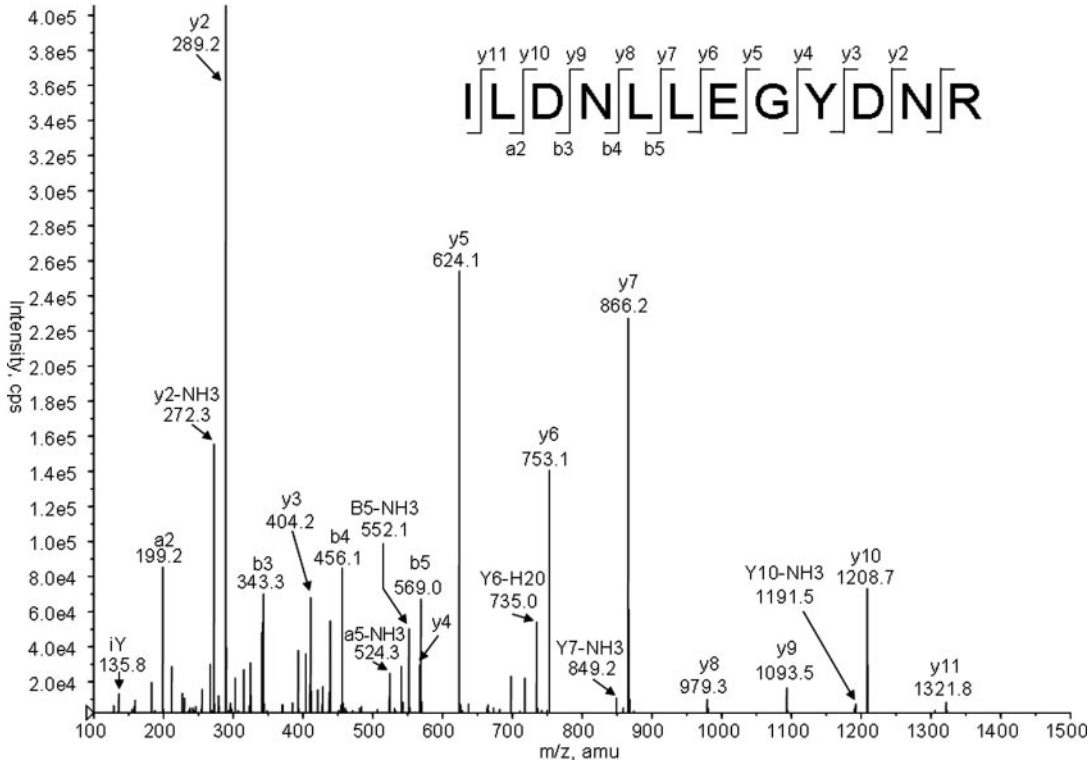

FIG. 3. **MS/MS-spectrum of the GABRA6 peptide ILDNLLEGYDNR.** A Mascot search against the Swiss-Prot database with the species subset *Rattus* identified the peptide ILDNLLEGYDNR with an elution time of 32.45 min as unique to GABRA6. The peptide was derived from a doubly charged precursor ion (m/z = 717.91)

neurotransmitter receptors and transporters. One of the receptors, GABRA6 (Fig. 3), is highly restricted to cerebellar granule cells (37). This demonstrates the potential to identify brain area-specific proteins by our approach. Another large group was comprised of proteins involved in cell-cell contact. Among the identified proteins were neurexin-3 and neuroligin-1, which play important roles in the formation and maintenance of the pre- and postsynaptic PMs (38). The postsynaptic protein neuroligin-1 was shown to induce presynaptic differentiation in axons (39). Neurexins are presynaptic interaction partners of neuroligins and induce postsynaptic specializations (40). Finally, we identified several proteins that play essential roles in neurological disorders, such as Alzheimer disease (amyloid βA4 protein) or the BSE-Creutzfeld-Jacob syndrome (major prion protein). The protocol may therefore foster the proteome analysis of neurological disorders either by characterizing known mutant proteins or by identifying novel markers for pathological conditions. An unexpected finding comprised the numerous proteins of the translation machinery. Ribosomal proteins represent a large fraction of the total protein mass of a cell, and their shear amount may explain their presence among the isolated proteins. An alternative explanation is provided by the existence of dendritic mRNA in cerebellar Purkinje cells, such as mRNA for calmodulin (41), inositol 1,4,5-trisphosphate receptor type 1 (42), and L7 (43). We conclude that the purified ribosomal proteins may be part of the dendritic translational machinery in the Purkinje cells, leading to their enclosure into the forming PM vesicles during tissue homogenization. In summary, we present here an easy to perform and rapid protocol that allows the preparation of highly enriched PMs from small brain areas. This will allow the proteome analysis of PMs of different brain regions, leading to improved understanding of the molecular repertoire underlying fundamental brain function and dysfunction.

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