Proteomics is potentially a powerful technology for elucidating brain function and neurodegenerative diseases. So far, the brain proteome has generally been analyzed by two-dimensional gel electrophoresis, which usually leads to the complete absence of membrane proteins. We describe a proteomic approach for profiling of plasma membrane proteins from mouse brain. The procedure consists of a novel method for extraction and fractionation of membranes, on-membrane digestion, diagonal separation of peptides, and high-sensitivity analysis by advanced MS. Breaking with the classical plasma membrane fractionation approach, membranes are isolated without cell compartment isolation, by stepwise depletion of non-membrane molecules from entire tissue homogenate by high-salt, carbonate, and urea washes followed by treatment of the membranes with sublytic concentrations of digitonin. Plasma membrane is further enriched by density gradient fractionation and protein digested on-membrane by endoproteinase Lys-C. Released peptides are separated, fractions digested by trypsin, and analyzed by LC-MS/MS. In single experiments, the developed technology enabled identification of 862 proteins from 150 mg of mouse brain cortex. Further development and miniaturization allowed analysis of 15 mg of hippocampus, revealing 1,685 proteins. More than 60% of the identified proteins are membrane proteins, including several classes of ion channels and neurotransmitter receptors. Our work now allows in-depth study of brain membrane proteomes, such as of mouse models of neurological disease.

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Selective spatially and temporally controlled expression and turn-over of proteins reflects tissue-specific differentiation and response to environmental stimuli. Individual regions of the brain, in particular across its various developmental stages and pathologic disorders, are likely to have distinct protein composition. Therefore, analysis of the brain proteome and its abnormalities would be very desirable in understanding the molecular basis of brain functioning and the etiology of its diseases. Recently, several proteomic profiling studies on entire brain, its compartments, and organelles have been reported (for a review see Ref. 1). A prevailing majority of that work was performed using two-dimensional electrophoresis (2DE) for separating proteins prior to mass spectrometric analysis, whereas only few studies were based on gel-free technologies.

Until now, application of 2DE for separation of proteins prior to mass spectrometric analysis enabled identification and relative quantification of 466 proteins from whole mouse brain cytosol (2). In total, 437 proteins were identified in mitochondria, microsomal, and cytosolic fractions of the rat brain (3). In another studies, 30, 38, and 165 different proteins were identified from mouse cerebellum (4), human pituitary (5), and human hippocampus (6), respectively.

Several groups focused their proteomic studies on proteomic profiling of the postsynaptic densities of rat brain (7–9). Yoshimura (8) identified 492 proteins using LC-MS/MS, whereas the two other approaches identified far fewer proteins. Recently, application of LC-MS/MS for comparative analysis of fractions enriched in plasma membranes (PM) from mouse fore- and hindbrain resulted in identification of 355 and relative quantification of 281 proteins (10).

In studies analyzing changes potentially contributing to human brain diseases performed using the 2DE technology, a significant number of up- and down-regulated proteins has been identified (for a review see Ref. 11). Most of them, however, represent highly abundant components with housekeeping function in neural cells. In contrast, many brain proteins of primary interest are less-abundant transmembrane and membrane-bound proteins, including neurotransmitter receptors, ion channels, and G-proteins. So far, effective methods for analysis of brain membrane proteins have not been established. In particular, techniques for efficient identification of PM proteins from small amounts of frozen tissue, which are typical clinical samples, have not been developed.

Identification and characterization of integral and PM-associated proteins is pivotal in the discovery of novel disease markers and drug targets. There are various methods for isolation of fractions enriched in PMs from cultured cells and tissue. Usually, they utilize mechanical or osmotic disruption of cells to generate cell subcomponents that vary in size and

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buoyant density and that are then separated by centrifugation techniques. These approaches are not ideal for frozen samples because the freezing process often affects organelles integrity. In particular, DNA released from broken nuclei often causes aggregation of other organelles that can be detrimental to the isolation of PMs. Moreover, homogenization-related formation of artifact membrane vesicles containing different cell components, as for example synaptosomes containing mitochondria, is a serious separation issue (12). To circumvent these preparative problems, we have developed an alternative approach for isolation of membranous material from tissue and purification of PM. The method is based on high-speed shearing of tissues in solution, enabling removal of a majority of soluble proteins. The resulting membranous material is separated by density gradient centrifugation that yields fractions enriched in PM proteins. The method is simple and can be applied to the isolation and comprehensive mapping of PM proteins from minute amounts of tissues, when coupled to a highly sensitive MS analysis.

In this study, we applied our membrane isolation method to proteomic profiling of cortex and hippocampus of mouse brain. Using limited amounts of frozen tissue, we were able to identify multiple members of ion channel, neurotransmitter receptor, and other PM protein families. Our approach offers a fast and sensitive method for identification of membrane proteins from brain and could become a basis for studying membrane proteins from various tissues and clinical biopsies.

**MATERIALS AND METHODS**

**Preparation and Fractionation of Membranes from Brain Tissue**—A total of 100–150 mg of frozen mouse brain cortex or 15–20 mg of hippocampus were blended in 1 ml of buffer A (2 mM NaCl, 10 mM HEPES/NaOH, pH 7.4, 1 mM EDTA) using an IKA Ultra Turbax blender at maximum speed of approximately 25,000 rpm for 30 s. The suspension was centrifuged in a Sorvall RC M150 GX using the S150AT rotor at 900,000 × g at 4 °C for 15 min. The supernatant was discarded and the pellet was re-extracted by blending as above in 4 ml (cortex) or 1 ml (hippocampus) of buffer B (0.1 mM Na₂CO₃, 1 mM EDTA, pH 11.3). After 30-min incubation on ice, the soluble material was removed by ultracentrifugation (as above). The pellet was extracted by blending in 1 ml (cortex) and 0.5 ml (hippocampus) of buffer C (4 mM urea, 100 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM EDTA). Digitonin was added to a final concentration of 2 or 4 mg/ml, and the suspension was incubated on ice for 30 min. The suspensions were mixed with Percoll (Amersham Biosciences, Piscataway, NJ) and 2 mM sucrose and buffered with buffer D (10 mM HEPES/NaOH, pH 7.4, 0.25 mM sucrose) in 11.5-ml (cortex) or 4-ml (hippocampus) tubes (Kendro Laboratory Products, Hanau, Germany). The final concentrations of Percoll and sucrose were 35% (v/v) and 0.25 M, respectively. The tubes were centrifuged at 50,000 rpm in the T 890 or S100AT6 rotor at 50,000 rpm at 4 °C for 32 or 28 min, respectively. The gradient was fractionated from the top by the displacement method. All buffers contained a complete protease inhibitor (Roche A/S Diagnostics, Hvidovre, Denmark).

γ-Glutamyl Transpeptidase and Protein Assays—Aliquots (5–20 μl) of collected fraction were incubated with 3 ml of assay solution containing 66 μM γ-L-glutamyl acid 7-amido-4-methylcoumarin (Glycosynth, Warrington, United Kingdom), 10 mM GlyGly, 10 mM MgCl₂, and 100 mM Tris, pH 9.0, at 37 °C for 10 min. Changes in fluorescence were recorded at excitation and emission wavelength of 365 and 460 nm, respectively. For protein determination, fraction aliquots were incubated at 96 °C in the presence of 20 mM SDS and 2 mM β-mercaptoethanol for 5 min. The insoluble material was removed by centrifugation at 20,000 × g from 10 min, and 1- to 5-μl aliquots of the supernatant were mixed with 3 ml of 8 M urea. Fluorescence was measured at excitation and emission wavelength of 295 and 360 nm, respectively. Tryptophanamide was used as a standard. The protein concentration was calculated assuming a mean tryptophan content in proteins of 1.3%.

**Protein Digestion, Carboxymethylation, and Reverse-phase Chromatography**—Fractions selected for further analysis were pooled and Percoll was removed by centrifugation at 900,000 × g at 4 °C for 15 min. The supernatant was discarded and the pellet resuspended in 1 ml (hippocampus) and 5 ml for (cortex) reduction buffer (100 mM Na₂CO₃, pH 11.5, 10 mM DTT). After 30 min of incubation on ice, the soluble material was removed by centrifugation as above. Pellet was resuspended in 1 ml (hippocampus) or 2 ml (cortex) of wash buffer (0.2 mM NaBr, 0.2 mM KCl, 50 mM Tris-HCl, pH 8.0, 10 mM DTT). After 30 min of incubation on ice, the soluble material was removed by centrifugation as above. Pellet was resuspended in 1 ml (hippocampus) or 2 ml (cortex) in 100 mM Na₂CO₃, pH 8.0. After 2 h of incubation at 25 °C, the soluble material was removed by centrifugation as above. Pellet was resuspended in 200 μl of 4 M urea, 100 mM Tris-HCl, pH 8.0. All resuspension steps were facilitated by sonication for 5 s. Five micrograms of endoproteinase Lys-C (Wako Bioproducts, Richmond, VA) were added, and the membranes were incubated overnight at room temperature. The released peptides were separated from the membranes by centrifugation as above and fractionated on a reverse-phase column and digested with trypsin as described previously (13).

**LC-MS/MS Analysis Using a Q-TOF Instrument**—Peptide fractions from the mouse cortex were analyzed using an LC Packings system (Dionex, Amsterdam, The Netherlands) coupled to a QSTAR Pulsar mass spectrometer (MDS Sciex, Toronto, Canada) as described (13). Data were acquired in information-dependent acquisition mode using the Analyst QS software, and only multiply charged ions were chosen for MS/MS. Each cycle was composed of 1-s MS and 2-s MS/MS. A Q2-pulsing function was applied.

**LC-MS/MS Analysis Using a Linear Ion Trap-FT Instrument**—Analysis of hippocampus samples were performed on a 7-Tesla Finnigan linear quadrupole ion trap-FT (LTQ-FT) mass spectrometer (Thermo Electron, Bremen, Germany) coupled to an Agilent 1100 LC (Agilent Technologies Inc., Palo Alto, CA) nanoflow system as described (14). Data were acquired in data-dependent mode using Xcalibur software—the three most intense ions detected in FT-ICR mode were isolated and fragmented in linear ion trap. For even more accurate molecular mass measurements, selected ion monitoring was used (14).

**Database Searches**—Database searches were performed using Mascot search engine (Matrix Science, London, United Kingdom) against mouse International Protein Index (IPI) protein sequence database (15). The sequences of common contaminations such as human and wool keratins, porcine trypsin, and endoproteinase Lys-C were added to the IPI database in FASTA format. For QSTAR datasets, the search parameters were: MS accuracy 0.2 Da, MS/MS...
accuracy 0.13 Da, allowed one miscleavage, fixed modification of
cysteine in carbamidomethylated form, variable modification of oxidi-
dized methionine and trypsin allowed to digest peptides containing
KP and RP. For the LTQ-FT dataset, required mass accuracy in MS
mode was 5 ppm and for MS/MS mode 0.5 Da.

Data Handling and Validation—The datasets retrieved by the MAS-
COT search engine were processed with MS QUANT (msquant.
sourceforge.net) and logged in the mass spectrometric data ware-
house—Experimental Peptide Identification Repository (EPIR), which
functions as a relational database (13). EPIR enables collection and
collapsing of results of database searches from the LC-MS/MS anal-
ysis, storage and mining MS/MS peptide evidence, and performing of
validation with an easy access to a selected spectrum. For manual
validation, two in-house-developed software tools, PEPSEA Inspec-
tor data validation tools and MS QUANT, were applied. All peptide
and protein data collected in our study are presented in the Supple-
mentary Table S1 and S2. Bioinformatics analysis was performed
using GeMiner (16).

RESULTS

Recent proteomic studies aimed at identification of mem-
brane proteins in brain required 1–15 g of tissue and resulted
in the identification of up to 500 proteins (3, 7–9). We have
previously employed a classical fractionation approach for
quantitative mapping of proteins in mouse fore- and hindbrain
with a total of 300 mg of tissue (10). Except cortex, all other
mouse brain compartments are smaller than 100 mg; there-
fore, to map proteins in distinct areas of mouse brain and to
avoid combining tissues from several animals, development
of an alternative approach is required. Moreover, to meet the
demands of clinical research, the amount of sample required
for comprehensive proteomic analysis should be significantly
reduced.

Here we break with the traditional subcellular fractionation
principle of separation of intact organelles. We avoid fraction-
aton of the biological sample by centrifugal sedimentation
into low speed (containing nuclei), medium speed (enriched in
mitochondria, lysosomes, and membrane vesicles), and high
speed (the so-called microsomal fraction). Instead of optimiz-
ing homogenization conditions preserving native state of or-
-ganelles, we focus solely on isolation of membranous mate-
rial. Finally, instead of separation of organelles utilizing their
differences in size, buoyant density, or charge, we fractionate
membranes by density gradient centrifugation.

Isolation of Membranes and Purification of PMs—Our ap-
proach consists of i) extraction of water-soluble proteins at
high-salt concentration, ii) extraction of extra-membranous
proteins from organelles and membrane vesicles, iii) density
gradient fractionation of the remaining membranous material,
and iv) on-membrane digestion with Lys-C followed by frac-
tionation and analysis of released peptides (Fig. 1).

The composition of biological membranes is variable and
their buoyant densities depend on the content of protein,
phospholipid, and cholesterol. As the composition is related
to the functional cell compartments, it can be exploited for
separation of membranes from various organelles. Density
gradient centrifugation of the membranous material in a self-
generating Percoll gradient revealed that membranes associ-
ated with PM marker activity γ-glutamyl transpeptidase were
more enriched in fractions of a slightly lower density than the
majority of other membranes (Fig. 2). Incubation of mem-
branes with sublytic amounts of digitonin according to the
procedure of Amar-Costesec et al. (17) improved separation
of PMs from other membranes (Fig. 2). Both concentrations of
digitonin tested resulted in similar changes in distribution of
marker activity and protein content.

Identification of PM Proteins from Mouse Cortex—To test
the applicability of the membrane preparation method to pro-
teomic analysis, membranes from a part of mouse cortex

Fig. 1. An overview on the procedure used in the identification
of membrane proteins from mouse brain tissues.
were prepared and fractionated on Percoll gradient. Two fractions with highest γ-glutamyl traspeptidase activity were combined, digested with endoproteinase Lys-C, and separated on a reverse-phase column. Sixteen fractions were collected, digested with trypsin, and each fraction was analyzed three times by LC-MS/MS on a Q-TOF instrument (QSTAR) using multiple exclusion lists. In this approach, a list of \( \text{m/z} \) values and retention time of sequenced peptides is created during the first run and the sample is then analyzed again but all of the previously detected and sequenced peptides are excluded. At least two additional LC-MS/MS analyses can be implemented to improve protein identification (13). Our analysis resulted in the identification of 8,274 peptides corresponding to 862 proteins (Table I). Analysis of the data using GoMiner revealed that 385 proteins had a known cellular localization and of these 66% were annotated as membrane proteins. Significantly, only 17 proteins were annotated as mitochondrial and 9 as endoplasmic membranes (Table I).

**Miniaturization of the Protocol—Identification of PM Proteins from Mouse Hippocampus**—The hippocampus is a part of the brain playing an important role in short-term memory and consolidation of long-term memory. In Alzheimer's disease, the hippocampus is one of the first regions of the brain to suffer attack; memory problems and disorientation appearing among the first symptoms. Despite the fact that it is one of the most studied regions of subcortical brain, only few efforts have been undertaken to analyze its proteome using 2DE (6, 18). Due to the well-known limitations of 2DE (1, 11), these approaches led to identification mostly of abundant enzymes and cytoskeletal proteins and did not reveal membrane proteins. To make our method applicable to identification of membrane proteins using minute amounts of biological material, we miniaturized the sample preparation protocol and applied a recently developed hybrid linear ion trap-FT mass spectrometer (LTQ-FT) (19). This instrument is significantly more sensitive, accurate, and has higher sequencing speed compared with a Q-TOF (20). Hippocampus from a single mouse (10–20 mg) was dissected from frozen brain. Fractions enriched in PMs were digested with endoproteinase Lys-C and separated on a reverse-phase column into eight fractions. These peptides were digested with trypsin and analyzed on the LTQ-FT. This resulted in the identification of more than 1,600 proteins. A total of 724 of these proteins could be assigned to subcellular localization with GoMiner: 60% were annotated as membrane proteins and of these only 24 were mitochondrial membrane proteins and 20 were endoplasmic-membrane proteins.

**Identification of Channel Proteins and Other Brain Disease-related Proteins**—The major neurotransmitters of the brain are glutamate and GABA that play excitatory and inhibitory functions, respectively. Our analysis enabled identification of a
significant number of the major classes of glutamate and GABA receptors (Table II). Identification of different isoforms and subunits depends on the peptide statistics and sequence coverage: the more peptides are detected the higher the chances to distinguish between variations in the sequence. For example, the three types of GABA receptors, A, B, and C, are expressed in many brain regions, but GABAC expression is restricted mainly to the retina. GABA receptors (Table II). mGluR1 was identified only in hippocampus, while GABAC receptors were identified by NR1, NR2A, and NR2B subunits. Kainate receptor GluR 6 was identified only in the hippocampus sample, which probably reflects high expression of its gene in the CA3 region of hippocampus as well as higher sensitivity of the LTQ-FT instruments. Two metabotropic receptors, mGluR3 and mGluR5, were identified in both analyses (Table II). mGluR1 was identified only in hippocampus, where it is preferentially located.

In addition to proteins constituting neurotransmitter-gated channels, our method revealed the presence of different voltage-gated cation channels (Tables S1 and S2). Subunits representing a, c, d, and q types of the K+−channels were identified. Voltage-gated Na+−channels consist of an α-subunit responsible for selectivity and voltage gating. However, some Na+−channels also have one or two smaller subunits called β-1 and β-2. We found three different α-type polypeptides as well as both β subunits. Voltage-gated Ca2+−channels regu-
late the flow of Ca\textsuperscript{2+} in excitable membranes. They are composed of four subunits: \(\alpha_1\), \(\alpha_2\delta\), \(\beta\), and \(\gamma\). Proteomic analysis enabled identification of representatives for all these subunits including three different members of the \(\alpha_2\delta\) type.

In our study, we were able to identify BSE-Creutzfeldt-Jacob syndrome major prion protein, which is a cell-surface glycoprotein anchored by a glycosylphosphatidylinositol moiety (21). We found the amyloid \(\beta\AA4\) protein and cell-surface receptor, mutations of which are related to Alzheimer (22), dementia, and severe cerebral amyloid angiopathy (23). Other disease-related proteins found comprise defender against cell death 1 protein and programmed cell death 6 interacting protein that are involved in apoptosis. Defender against cell death 1 protein is a hydrophobic protein bearing sequences of potential transmembrane domains.

**DISCUSSION**

Usually biological samples contain thousands of distinct proteins that cannot be separated solely using one- or two-dimensional separation techniques prior to mass spectrometric analysis. We previously demonstrated in-depth proteomic studies of organelles such as mitochondrion (24), the nucleolus (25), and the centrosome (26). In this study, we focused on one of the most valuable cellular compartment for molecular biology and drug discovery—the PM. Our approach combines a new PM purification method, digestion of membrane proteins on nonsolubilized membranes (10, 27), diagonal chromatography, and highly sensitive MS. The novel method for enrichment of PMs from frozen tissues has been applied to mapping of cortical and hippocampal membrane proteins. In both experiments, a substantial enrichment of membrane proteins was achieved—about 60% of identified proteins were membrane proteins. This enabled identification of a significant number of receptor, ion channel, and other integral PM proteins. Vice versa, the contamination of the PMs by mitochondrion, an abundant organelle with highly abundant membrane proteins, was relatively low (\(\sim 5\%\) of membrane proteins).

Advantages of digestion of integral membrane proteins of intact membranes have been demonstrated recently (10, 27). The membrane can be considered as a solid phase carrying polypeptides that can be modified while immobilized. Disulfide bond reduction, thiol alklylation, and digestion of the polypeptides is facilitated in this way. Recently, we have demonstrated that this procedure can be applied to modification of thiol-containing peptides for complexity reduction and relative quantification of membrane proteins by the Hys-Tag reagent (10).

Multidimensional chromatography is an effective way to resolve very complex peptide mixtures (28). In the current study, off-line LC fractionation of endopeptidase Lys-C peptides was followed by trypsin digestion and LC-MS/MS analysis. Commonly employed 2D LC-MS/MS technology with low-resolution ion trap mass spectrometers (27–30) frequently introduces low-confidence database hits and results in difficulties in data interpretation. In contrast, we employed either a Q-TOF or a linear ion trap FT mass spectrometer. The analysis of cortex samples was performed on a Q-TOF instrument. In order to extract maximum information and resolve the complexity of the PM proteome, we analyzed a significant number of fraction using exclusion lists.

In principle, FT-ICR instruments can provide extremely high resolution and parts per million (ppm) mass accuracy, but until recently this technology was not sufficiently mature for routine large-scale proteomics applications. With the advent of the LTQ coupled to FT-ICR cell, these limitations have been overcome resulting in precursor mass accuracy around 1 ppm, several fragmentation events per second, and exquisite sensitivity in the subfemtomole range. This technology, combined with the novel PM preparation method, has allowed us to probe the brain membrane proteome to great depth. In the analysis of membrane fractions of hippocampus, the LTQ-FT detected 1,685 proteins using 10 times less material compared with the analysis with a Q-TOF instrument. Comparison of cortex and hippocampus datasets using the EPIR comparison module showed that 613 protein groups were common for both cortex and hippocampus samples.

Clearly, we were able to cover many of the membrane proteins of interest in brain proteome studies. In a previous study (10) that was concerned with quantitative profiling of proteins from mouse fore- and hindbrain, we needed >100 mg pieces of tissue for preparation of PM material and therefore we had to combine material from several animals. Now, applying the new purification technique and more sensitive mass spectrometric technology, it is possible to profile and quantify proteins from specific brain areas, such as hippocampus and cerebellum, without mixing tissue from different animals, thus unraveling their natural variability between individuals. As the majority of clinical samples are biopsies of size of few milligrams or less, the demand for proteomic methods suitable for analysis of small amounts of tissue is obvious. Our approach offers a method for comprehensive profiling of proteins from isolated membranes or enriched PMs requiring minute amounts of tissue in range of few milligrams. The combination of relatively simple sample preparation technique with high-sensitivity and high-confidence MS opens new possibilities in studying membrane proteins and brain proteome. It extends perspectives for proteomic-driven research aimed at unraveling of mechanism of neurological diseases, memory disorders, and aging processes.

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Proteomic Mapping of Brain Plasma Membrane Proteins

[The on-line version of this manuscript (available at http://www.mcponline.org) contains supplemental material.

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