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Optimized procedure of extraction, purification and proteomic analysis of nuclear proteins from mouse brain

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HIGHLIGHTS

- An optimized method for mouse brain nuclear proteome analysis is presented.
- Sonication, nuclease digestion and protein precipitation with acetone are required.
- The procedure provides high quality two-dimensional electrophoresis maps.
- Shotgun mass spectrometry analysis reveals enrichment in nuclear proteins.

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ABSTRACT

Background: The cell nucleus is a highly dynamic subcellular compartment performing crucial processes for functioning and administration of the cell. Proteomic analysis of isolated nuclear fraction enables in-depth insight into these processes leading to better understanding of physiological and pathological state of the brain. There is no universal method for nuclear proteome investigation and each biological material needs individual optimization. An additional difficulty is the large amount of nucleic acids, which impairs isoelectrofocusing of proteins and shotgun mass spectrometry analysis of complex peptide samples.
New method: We performed the first comprehensive optimization of intact nuclei isolation from mouse brain in combination with nuclear protein purification prior to two-dimensional gel electrophoresis (2DE) and gel-free proteomic analysis.
Results: Application of sonication, digestion with nuclease and protein precipitation with acetone allowed to obtain high quality 2DE gels. Shotgun mass spectrometry analysis of isolated proteins proved an enrichment in nuclear proteins. The 66.4% of 265 identified proteins had assigned *nucleus* localization in UniProt database. Gene Ontology analysis using DAVID platform revealed the highest fold enrichment in spliceosome (24.5), nuclear periphery (12.4) and nuclear matrix (11.3).
Comparison with existing methods: The proposed procedure is tailored to mouse brain tissue nuclear subproteome investigation. The quality of isolated nuclei, the effectiveness of the protein purification, efficiency of protein recovery after precipitation and overall method reproducibility was taken into detailed consideration.
Conclusions: The elaborated procedure could be further applied for in-depth proteomic analysis of molecular processes occurring in the mouse brain nucleus.

1. Introduction

The cell nucleus is a key organelle of eukaryotic cell. Its functions extend far beyond genetic information storage, gene expression and RNA splicing (Tripathi and Prasanth, 2011). It acts as a command center of the cell and impacts on regulation of transcription, cytoskeletal organization, cell migration and signaling pathways (Burke and Stewart, 2014). In order to shed light on functioning of the nucleus in health and disease, proteomic methods are often employed (Beausoleil et al., 2004; Jung et al., 2000; Turck et al., 2004; Zhang et al., 2006). Nuclear proteins are estimated to

Abbreviations: IEF, isoelectrofocusing; IPG, immobilized pH gradient; FPR, False Positive Ratio; GO, Gene Ontology; DAVID, database for annotation visualization and integrated discovery; 2DE, two-dimensional gel electrophoresis; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; nanoHPLC, nano high-performance liquid chromatography; PCIA, phenol/chloroform/isoamyl alcohol; TCA, trichloroacetic acid.

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make up approximately 20% of the animal proteome, but in proteomic experiments they are usually masked by highly abundant metabolic enzymes and structural proteins (Narula et al., 2013). The most interesting regulatory molecules are often below the detection limit in total cell lysates (Gatto et al., 2010), let alone the transcription factors, which are the least abundant proteins in eukaryotes (Henrich et al., 2007). Analysis of the organelle fraction reduces the complexity of tissue extracts facilitating more accurate mapping of the nuclear proteins. In such experiments, it is critical to obtain a nuclei fraction of high purity, what is not trivial. Too gentle homogenization may lead to the formation of organelle aggregates, while harsh homogenization can result in nuclei damage. It is important to deliberately choose a procedure among many described protocols of organelle isolation and protein purification (Cox and Emili, 2006). Appropriate method for a particular biological material should be applied.

Another important issue is to select proper research method for analysis of content and quantity of nuclear proteins. Recently rapid development of mass spectrometry has triggered rise in popularity of gel-free approach in proteomic researches. Nevertheless, two-dimensional gel electrophoresis (2DE) is still the most commonly used technique in top-down proteomic studies (Silva et al., 2014). Application of 2DE approach enables observing protein isoforms, including post-translational modifications, which control the signaling pathways and the synaptic proteins activity. Post-translational modifications of histone proteins, such as phosphorylation, acetylation, methylation and ubiquitination modulate the chromatin structure and affect the binding of transcription factors to the DNA (Tweedie-Cullen and Mansuy, 2010). Usage of 2DE parallel with high-throughput shotgun analysis as a complementary method allows deeper characterization of a sample (Oliveira et al., 2014) and such an approach we propose in the current study. Moreover, regardless gel-based or gel-free proteomic approach is considered to be further applied, the use of 2DE at the stage of subfractionation optimization allows to accurately monitor presence of contaminations and evaluate the reproducibility of the whole procedure.

Until now, several 2DE maps of nuclear proteins isolated from various biological material have already been published, but none of them from mouse or rat brain (Baumeister et al., 2001; Buhr et al., 2008; de Mateo et al., 2011; Henrich et al., 2007; Jung et al., 2000; Pandey et al., 2008; Shakib et al., 2005; Skupien-Rabian et al., 2016). Low quality of some of the reported protein separations confirms, that nuclei pose difficult material for 2DE. This is because large amounts of nucleic acids nonspecifically bind to positively charged proteins. Consequently, proteins have widely varying charges, that causes their inhomogeneity resulting in horizontal streaking in the acidic part of the gels (Gorg et al., 1997). In the abovementioned papers every sample was prepared in a different way, which indicates that there is no universal method for nuclear proteome investigation and each biological material needs individual optimization, both in terms of a homogenization step (Huber et al., 2003) and purification of proteins (Fic et al., 2010).

In the present study, we show the first comprehensive optimization of sample processing prior to 2DE and shotgun analysis for mouse brain nuclei. We tested different homogenizers and various conditions of nuclear protein purification in order to obtain good quality of 2DE separation and successful shotgun analysis. We also aimed to validate of the enrichment in nuclear proteins by Gene Ontology (GO) analysis. Proposed method can be applied in further qualitative and quantitative studies to examine neurochemical changes in psychiatric disorders, drug/alcohol addiction in common used mouse models (Seong et al., 2002). Well-defined procedure for nuclear proteome investigation may also provide information about protein location and define new moonlighting proteins (Jeffery, 2005).

2. Materials and methods

2.1. Reagents and chemicals

Acetic acid, acetone, ethanol, chloroform, HCl, methanol, glycerol and formaldehyde were purchased from POCh (Poland). Acrylamide, bis-acrylamide, CHAPS, urea, thiourea, bromophenol blue, DTT, iodoacetamide, SDS, $CaCl_2$, ammonium persulfate, sodium carbonate, sodium acetate, sodium thiosulfate, sucrose, glycine, agarose, Tris base, PMSF, magnesium chloride hexahydrate and silver nitrate were purchased from BioShop Canada Inc. (Canada). Complete Protease Inhibitor Cocktail Tablets were from Roche (Germany). PPS Silent Surfactant was purchased from Protein Discovery, Inc/Expedeon (USA). Benzonase® Nuclease, phenol:chloroform:isoamyl alcohol (25:24:1), EDTA, trypan blue, potassium phosphate and Triton® X-100 were purchased from Sigma-Aldrich (USA). Immobilized pH gradient (IPG) strips 3-10NL 7 cm, IPG Buffer pH 3-10NL and Drystrip Cover Fluid were acquired from GE Healthcare Life Sciences (Sweden). Ammonium bicarbonate and trichloroacetic acid (TCA) were from Fluka. Vivaspin 500 VS0111 were acquired from Sartorius (Germany). Sequencing Grade Modified Trypsin was purchased from Biocentrum (Poland). All high-performance liquid chromatography (HPLC) solvents were purchased from JT Baker (USA). Reversed Phase HPLC columns were from Thermo Fisher Scientific (USA).

2.2. Animals

The experiments were carried out on mouse brains. The animals were killed by cervical dislocation, the brains were rapidly dissected, divided into cerebral hemispheres, immediately frozen and stored at −80 °C until analysis. The experiments were performed in accordance with the regional legal regulations.

2.3. Nuclei purification

Nuclei were isolated from the mouse brain as described by Lovtrup-Rein and McEwen (Lovtrup-Rein and McEwen, 1966) with minor modifications. All operations were performed at 0–4 °C. The cerebral hemispheres (approximately 0.25 g) were homogenized by hand in Potter–Elvehjem Tissue Grinder (clearance 150÷250 μm Cole Parmer) in 3.75 ml of buffer A (320 mM sucrose, 1 mM $MgCl_2$, 1 mM K_3PO_4, 0.25% Triton X-100, pH 6.5, 1 mM PMSF). The homogenates obtained after 12 strokes were filtered through cheesecloth and centrifuged for 10 min at 850×*g*. The supernatants were discarded, the pellets were resuspended in the same volume of buffer A without Triton X-100, then centrifuged for 10 min at 850×*g*. The pellets were washed again in the same way and centrifuged for 8 min at 600×*g*. The crude nuclear pellets were resuspended in 2.7 ml of buffer A without Triton X-100, added to 13.8 ml of buffer B (2.23 M sucrose, 1 mM $MgCl_2$, 1 mM K_3PO_4, pH 6.5, 1 mM PMSF) and mixed precisely. The suspensions were centrifuged for 2 h at 53,500×*g* in the swinging-bucket rotor (SureSpin 630, 17 ml) in Sorvall WX Ultra 80 ultracentrifuge (Thermo Scientific). The purified nuclei pellets were obtained as a small sediment at the bottom of the centrifuge tube and the cytoplasmic debris floated on hypertonic buffer.

2.4. Microscope observation

The nuclear pellets were resuspended in 300 μl of buffer A without Triton X-100 and stained with trypan blue. The nuclei were observed with an inverted Leica DMI6000 B microscope equipped with integrated modulation contrast optics.

2.5. Extraction of proteins from nuclei

Two methods of nuclear protein extraction were tested: with and without Benzonase addition. The procedure without the use of nuclease is described in Supporting Information A (S1). Below we present the preferable protocol with nuclease addition.

The purified nuclei pellets were resuspended in 200 μl of lysis buffer (5 M urea, 2 M thiourea, 2% CHAPS, 40 mM Tris-HCl pH 8.5, 1 mM $MgCl_2$, 65 mM DTT, 1 mM PMSF) with 10 U of Benzonase and incubated on ice for 5 min. The samples were sonicated twice on ice for 15 min (320 W, 30 s on/off) using Bioruptor™ UCD-200 (Diagenode, Liege, Belgium). Afterwards, 40 U of Benzonase was added to the protein samples and incubated at 25 °C for 25 min. The lysates were centrifuged at 20,000×*g* for 10 min at 25 °C, the pellet was resuspended in 50 μl of lysis buffer and spun (20,000×*g* for 10 min at 25 °C) and the supernatants were pooled. The protein concentration was determined by Bradford assay (Bradford, 1976). Aliquots (150 μg) of the protein mixture were used to test precipitation procedures.

2.6. Protein precipitation

Proteins extracted from the nuclei were cleaned up using different precipitation methods: methanol/chloroform, ethanol, phenol/chloroform/isoamyl alcohol, TCA, as well as purification using Vivaspin columns. The protein recovery was determined as a ratio of the protein amount before and after precipitation.

2.6.1. Purification of protein samples using Vivaspin 500 (cut off 10 kDa)

Lysate was centrifuged in the Vivaspin 500 at 4500×*g* for 25 min at 12 °C. The procedure was repeated five times, by adding the same volume of 8 M urea solution which flowed through the Vivaspin. Subsequently, the final protein concentrate was transferred to an eppendorf tube. Vivaspin was washed twice with 8 M urea solution. Protein concentrate and the two final washes were pooled together.

2.6.2. Methanol/chloroform precipitation

Precipitation was performed at room temperature. Four volumes of methanol were added to one volume of the protein sample and vortexed. One volume of chloroform was added and the mixture was vortexed. Three volumes of distilled water were added and then vortexed. The sample was centrifuged for 10 min at 10,000×*g*. The upper layer was carefully removed without disturbing the precipitate at the interphase. Four volumes of methanol were added and vortexed. After that, the sample was centrifuged for 10 min at 10,000×*g*. The supernatant was discarded, and the protein pellet was air dried.

2.6.3. Ethanol precipitation

Nine volumes of ice-cold ethanol were added to one volume of protein sample and vortexed. Precipitation was carried out overnight at −20 °C. After that, the mixture was centrifuged at 16,000×*g* for 15 min at 4 °C. The supernatant was removed and the protein pellet was air dried.

2.6.4. Phenol/chloroform/isoamyl alcohol (PCIA) precipitation

Two volumes of phenol/chloroform/isoamyl alcohol solution (25:24:1, v/v/v) were added to one volume of the protein sample and vortexed. The mixture was incubated for 5 min and then centrifuged at 21,000×*g* for 10 min at 4 °C. The proteins should be at the interphase between aqueous phase saturated with isoamyl alcohol and organic phase. Both aqueous and organic phase were carefully removed. 1 ml of ice-cold acetone was added to the sample, vortexed and then centrifuged at the same conditions as before. The protein pellet was air dried.

2.6.5. TCA precipitation

Precipitation was performed at 4 °C. A 20% (w/v) solution of TCA with 20 mM DTT was added to the protein sample to a final concentration of 15% and the mixture was vortexed. The sample was incubated for 2.5 h on ice and then centrifuged for 10 min at 10,000×*g*. Afterwards, the protein pellet was washed twice with 1 ml of ice-cold acetone. The supernatant was removed and the protein pellet was air dried.

2.6.6. Acetone precipitation

Seven volumes of ice-cold acetone were added to one volume of the protein sample and vortexed. The mixture was incubated for 1.5 h at −20 °C. Subsequently, the sample was centrifuged at 20,000×*g* for 15 min at 4 °C. The supernatant was discarded and the protein pellet was air dried.

2.7. 2DE

After precipitation, the protein pellets were solubilized in rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.002% bromophenol blue). The protein concentration was determined by Bradford assay. 2DE was conducted according to the previously published protocol (Fic et al., 2010). Samples that contained 30 μg of nuclear proteins were filled up to 125 μl of rehydration buffer with 20 mM DTT and 1% IPG buffer added just before use. IPG strips (7 cm non-linear, pH 3–10) (GE Healthcare) were passively rehydrated with protein samples for 16 h covered with Immobiline DryStrip Cover Fluid. IEF was performed using Amersham Ettan IPGphor 3 IEF system (GE Healthcare, Uppsala, Sweden) at 20 °C with a current limit 50 μA per strip. The following voltage steps were applied: 100 V for 2 h, a gradient to 300 V for 0.5 h, a gradient to 1000 V for 0.5 h, a gradient to 5000 V for 1.5 h and 5000 V for 1 h. Between electrodes and strips the paper wicks were placed, soaked with rehydration buffer containing 20 mM DTT. During prefocusing the paper wicks were exchanged twice. After focusing, the IPG strips were equilibrated in two steps for 15 min using equilibration buffer (75 mM Tris-HCl, pH 8.8, 30% glycerol, 6 M urea and 2% SDS) with additional 1% (w/v) DTT in the first step and with 2.5% (w/v) iodoacetamide in the second step of equilibration. Subsequently, the proteins were separated on Mini-Protean 3 (BioRad) using 12% polyacrylamide gels at 70 V for 25 min and at 170 V. The protein pattern was visualized by silver staining (Yan et al., 2000) and scanned using Image Scanner (GE Healthcare). Image analysis was conducted in Image Master 2D Platinum v6.0 software (GE Healthcare).

2.8. Mass spectrometry shotgun analysis of nuclear proteins

To determine the protein composition of the obtained fractions, the procedure of nuclei isolation was repeated twice, each time starting from the cerebral hemisphere. Nuclear proteins were extracted with addition of Benzonase as described above. The protein pellets (approximately 100 μg) after acetone precipitation were resuspended in 50 mM ammonium bicarbonate and 0.1% (w/v) PPS Silent Surfactant according to the manufacturer's instructions. To improve the solubility of proteins, the samples were sonicated on ice for 15 min (200 W, 30 s on/off) using Bioruptor™ UCD-200. Subsequently, reduction and alkylation of proteins were performed. DTT was added to a final concentration of 5 mM and incubated for 30 min at 50 °C. Next, the samples were cooled to

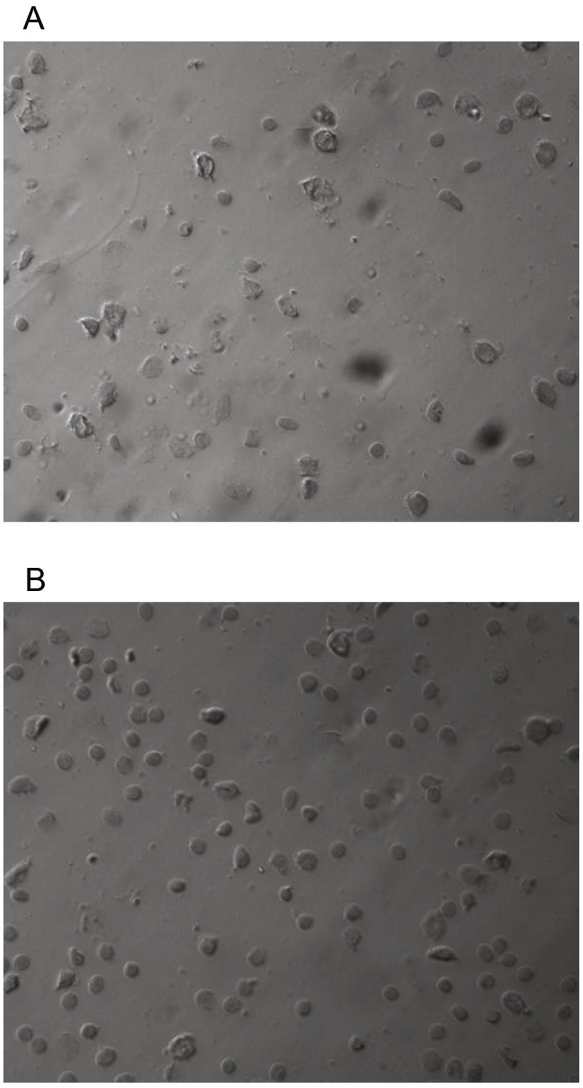

Fig. 1. Representative pictures of the isolated nuclei from mouse brain stained by trypan blue. Tissue was homogenized using Potter–Elvehjem Tissue Grinders, clearance: 110–150 μm (A), 150–250 μm (B). Isolation with loose-fitting pestle homogenizer (B) yielded pure and intact nuclei extract.

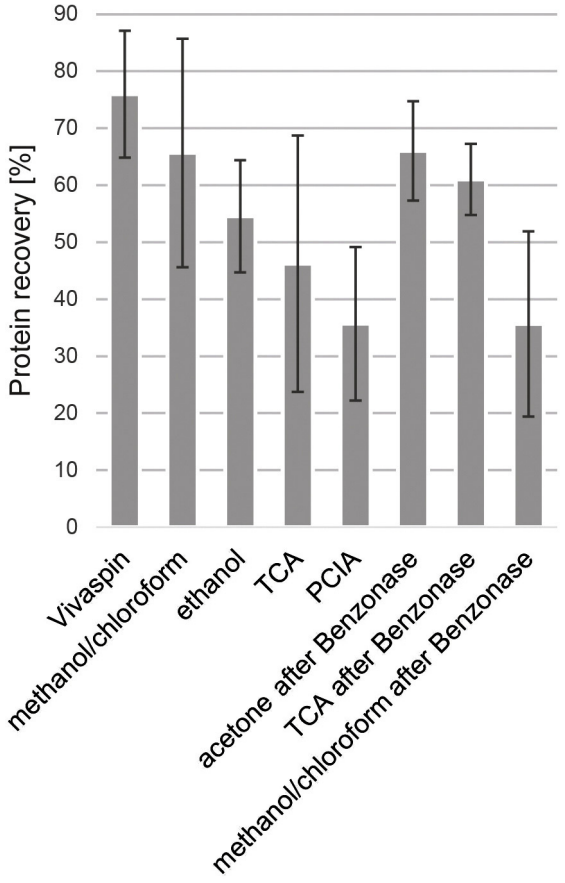

Fig. 2. Comparison of the average values of the protein recovery yields of different protein purification methods from mouse brain nuclei. Error bars represent the standard deviation from at least three replicates.

room temperature. Iodoacetamide was added to a final concentration of 15 mM and incubated for 30 min at room temperature. Then, $CaCl_2$ was added to the mixture to a final concentration of 1 mM. Trypsin was added in enzyme:protein ratio of 1:50 and incubated overnight at 37 °C. The reaction was stopped by addition of HCl to a final concentration of 200 mM and incubation for 45 min at 37 °C. The sample was centrifuged for 10 min at 16,000 × *g* to remove insoluble material.

The supernatants were analyzed on a micrOTOF-QII (Bruker Daltonics) coupled with a UltiMate 3000 RSLCnano System (Dionex). The samples were loaded onto a trap column (AcclaimPepMap100 C18, ID 75 μm, length 20 mm, particle size 3 μm, pore size 100 Å) in 0.05% TFA, 2% ACN at a flow rate of 5 μl/min and resolved on an analytical column (AcclaimPepMapRLSC C18, ID 75 μm, length 150 mm, particle size 2 μm, pore size 100 Å) at a flow rate 300 nl/min. Peptides were eluted with a 120 min gradient ranging from 2% to 40% ACN in 0.05% formic acid/water. MS/MS data were acquired by targeting 5 precursor ions with dynamic exclusion set to 30 s.

The raw data were processed by Data Analysis 4.1 (Bruker Daltonics) and searched using locally installed MASCOT (v.2.5.1, Matrix Science) embedded into ProteinScape 3.0 (Bruker Daltonics) against the SwissProt_201508 database with taxonomy restriction to Rodentia (26,337 sequences). The following search parameters were applied: digestion by trypsin with maximum one missed cleavage; precursor and product ions mass tolerance respectively 20 ppm and 0.05 Da; carbamidomethylation (C) as fixed modification; oxidation (M) and deamidation (NQ) as variable modifications. Peptides above score threshold 15 were accepted. The False Positive Ratio (FPR) was estimated by searching against decoy database and was set to below 1% at the protein level. The resulting two lists of proteins were compiled using ProteinScape software. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [38] via the MassIVE repository with the dataset identifier PXD002872.

2.9. Enrichment Gene Ontology (GO) analysis using shotgun proteomics

Cellular localization of the identified proteins was studied using *subcellular location* term assigned to particular protein in UniProt database. The obtained distribution of protein localization was compared to distribution in the *Mus musculus* proteome from UniProt/SwissProt database (released December 2014, 16,677 reviewed sequences).

GO enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID), v.6.7 (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009). The results were filtered by the following criteria: *p*-value (EASE score) <0.01 and proteins counts >5.

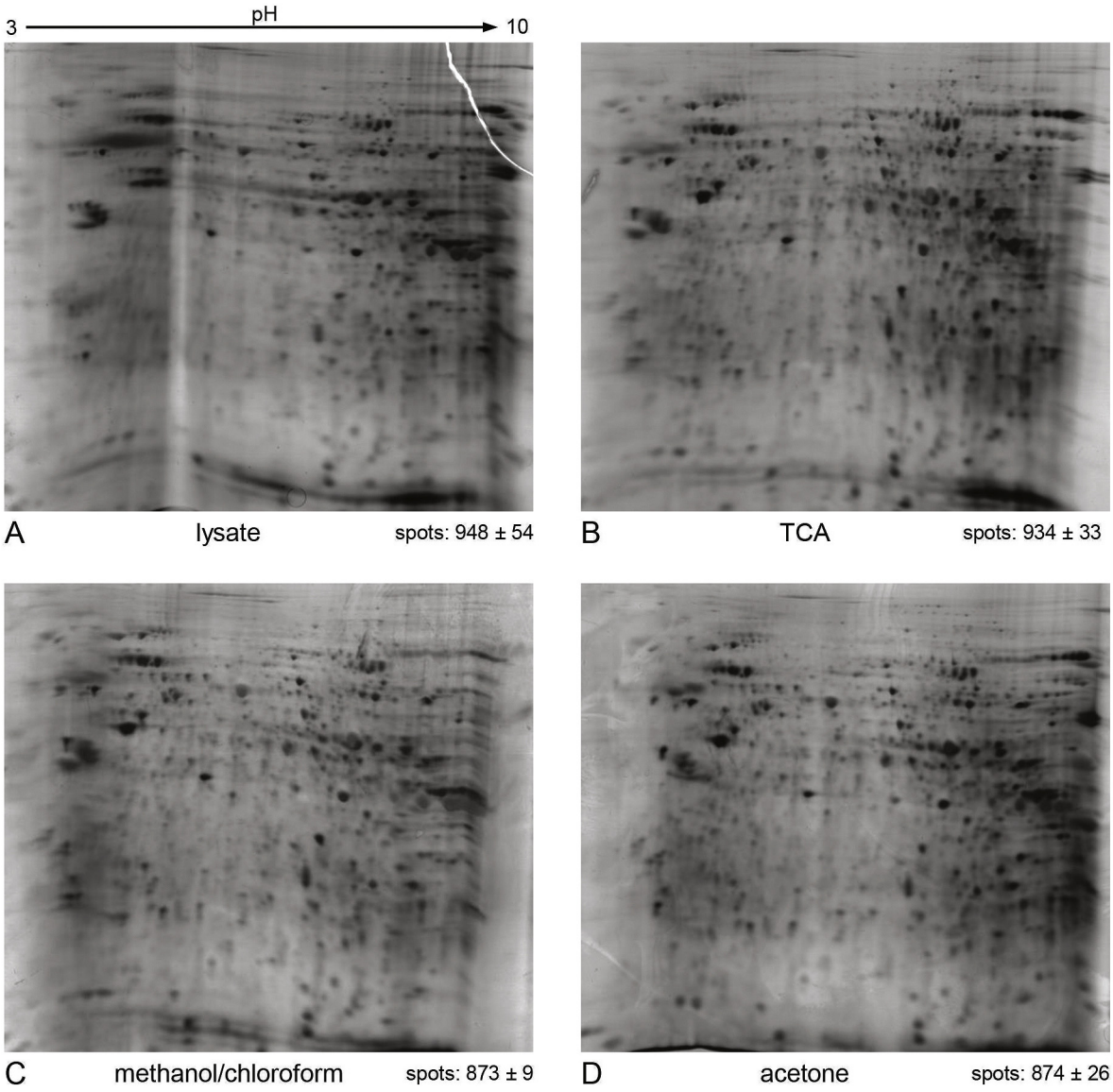

Fig. 3. Representative 2DE images of mouse brain nuclear proteins prepared with Benzonase addition. Samples were separated on 7 cm IPG strips pH 3–10 and 12% SDS-PAGE gels: nuclear lysate without precipitation (A), nuclear proteins purified by various precipitation methods: TCA (B), methanol/chloroform (C), acetone (D). Average number of spots detected on three gel replicates is presented. Proteins were visualized by silver staining.

2.10. Statistical analysis

In order to evaluate reproducibility of the sample preparation procedures, each precipitation followed by 2DE separation was repeated three times. From the replicates the average protein recovery with the standard deviation were calculated. Mass spectrometry shotgun analysis was performed in two biological replicates.

3. Results and discussion

Precise organelle isolation together with proper protein extraction are essential to perform reliable comparative high-throughput experiments, both gel-based and gel-free. This study aimed to optimize method for intact nuclei isolation from mouse brain in combination with nuclear protein purification prior to proteomic analysis. Various parameters of the procedure were tested and series of 2DE were performed to reveal the best method of brain subproteome investigation. During optimization, the following issues were taken into account: effectiveness of the protein purification, efficiency of protein recovery after precipitation and procedure reproducibility.

3.1. Intact nuclei isolation procedure

Nuclei from brain are extremely fragile (Sporn et al., 1962), so it is essential to precisely adjust conditions of homogenization step. For nuclei isolation the most commonly used buffers are based on sucrose or glycerol. We chose sucrose because glycerol (for example used by Shakib (Shakib et al., 2005)) has lower viscosity than sucrose and may permeate some organelles (Castle, 2001). We tested a homogenizer with a tight-fitting (clearance 110–150 μm) (Kislinger et al., 2006) and a loose-fitting pestle (clearance 150–250 μm) (Lovtrup-Rein and McEwen, 1966). Microscope observations of obtained nuclei samples showed that the procedure using tight-fitting pestle homogenizer resulted in greater damage of the organelles (Fig. 1A). Therefore, for nuclei isolation from mouse brain we recommend 12–14 strokes of homogenizer with clearance 150–250 μm, that yields pure and intact nuclei extract (Fig. 1B).

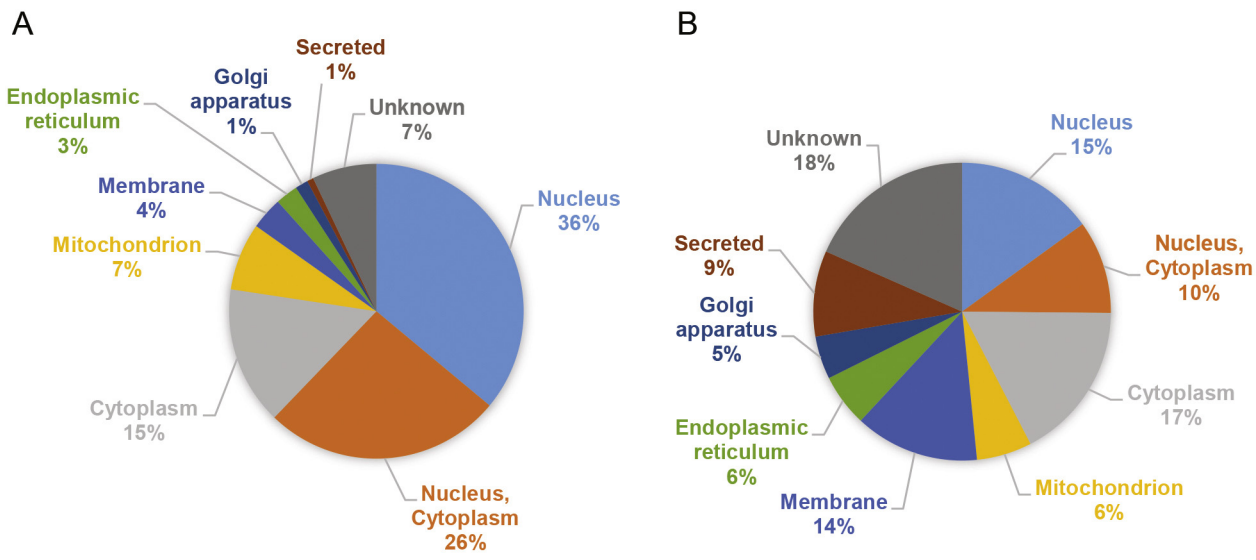

Fig. 4. Comparison of distribution of *subcellular location* annotations from UniProt database in set of 265 proteins identified from nuclei (A) and in the *Mus musculus* proteome (SwissProt database, released December 2014, 16,677 reviewed sequences) (B).

3.2. Comparison of different methods of nuclear proteins purification prior to proteomic analysis

Proteomic analysis is highly susceptible to interfering substances. Salts, detergents and lipids affect the 2DE pattern as well as hamper ionization and fragmentation process in MS-based shotgun analysis. In the case of nuclei, besides these common contaminants, the main problem is a large amount of DNA and RNA that form complexes with positively charged proteins. Moreover, in liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) experiments high sample viscosity caused by nucleic acid presence could impair the resolution of peptides during chromatographic separation. In order to disintegrate nucleic acid chains into short fragments and break the unspecific bounds, we applied high power ultrasonic waves during nuclear protein extraction, which should reduce disturbances in protein separation (Roy et al., 2014). In our hands, sonication significantly decreased the viscosity of the samples, which was observed while handling sample, however, it did not prevent streaking on 2DE gels.

Further attempts to remove DNA included precipitation of proteins using four different methods: ethanol, methanol/chloroform, phenol/chloroform/isoamyl alcohol (PCIA), TCA or purification using Vivaspin concentrators. The average protein recovery efficiency of three replicates after each tested method is shown in Fig. 2. To examine the effectiveness of the interfering substances removal, proteins were separated by 2DE. Obtained gel images are presented in Fig. S1 in Supporting Information A.

Method with the use of protein concentration on columns proposed by Xavier et al. (2010) was considered to be a promising alternative to the commonly used precipitation procedures, which usually do not provide full recovery of the proteins. Indeed, purification on Vivaspin concentrator resulted in the greatest protein recovery, but it did not clean the samples from contaminants, which disrupted IEF in acidic region. Worse effectiveness than showed by Xavier et al. may arise from higher nucleic acid concentration in nuclei lysate than in whole cell lysate. Nevertheless, the Vivaspin method after further optimizations may be considered as a potential method of sample preparation prior to gel-free analysis, which is less susceptible to ionic contaminants in comparison to IEF.

Precipitation with ethanol also did not remove interferences, which may have been caused by the tendency to precipitate proteins together with the nucleic acids (Xavier et al., 2010). Separations of proteins after methanol/chloroform and TCA precipitation were slightly better, but in the acidic region streaks were also present. The best purification effectiveness was obtained after PCIA precipitation. In alkaline environment majority of proteins is negatively charged and DNA-protein complexes disrupt (Antonioli et al., 2009). However, the PCIA method yielded very low protein recovery ($36 \pm 13\%$) that made this technique inappropriate for limited amounts of nuclear extracts.

Because none of the methods provides simultaneously high protein recovery and efficient removal of nucleic acids, we decided to apply enzymatic cleavage with Benzonase—an endonuclease which degrades all forms of DNA and RNA, but have no proteolytic activity. Step of incubation with Benzonase was added before sonication of the nuclear lysate. It should be emphasized that nuclease activity in strong denaturing buffer is attenuated. To enhance the action of Benzonase we modified lysis buffer by decreasing urea concentration and addition of required magnesium ions. Subsequently, the protein samples were precipitated with different agents: TCA, methanol/chloroform and acetone. Cleaned samples as well as nuclear lysate without precipitation were separated by 2DE. The representative 2DE gels with average number of detected spots are presented in Fig. 3.

The use of the nuclease significantly improved the quality of protein separation. The gel images obtained after all tested precipitations were almost free of streaking and the amounts of detected spots on gels were similar. The best protein recovery was achieved after acetone precipitation ($66 \pm 9\%$). Slightly worse recovery was observed after TCA precipitation ($61 \pm 6\%$) and very low after methanol/chloroform precipitation ($36 \pm 16\%$). Thus, all these findings brought us to the conclusion that the best method of nuclear sample purification prior to 2DE was acetone precipitation. It was characterized by high yield of protein recovery, good reproducibility and effectiveness in removing contaminants, what resulted in good quality of 2DE gels. Acetone precipitation was also used by Shakib to purify nuclear proteins from kidney fibroblasts what also provided good quality of 2DE separation (Shakib et al., 2005).

The proposed purification protocol of nuclear proteins omits dialysis step, which was used by some investigators (Baumeister et al., 2001; Shakib et al., 2005) hereby we minimized material losses and protocol complexity.

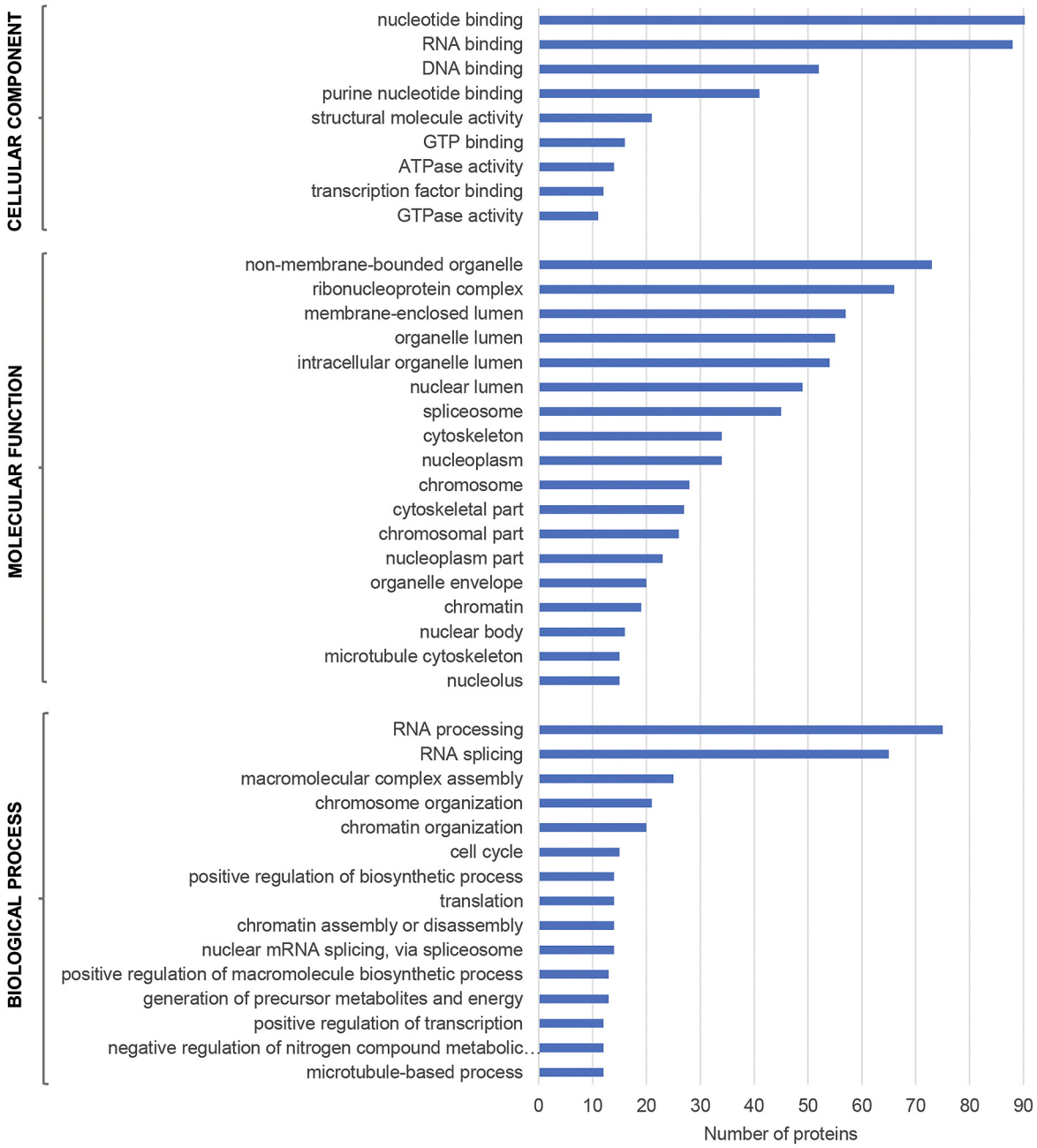

Fig. 5. Gene Ontology analysis of proteins isolated from nuclei by the DAVID platform. The most represented annotations in each GO category are showed. Detailed results are listed in Supporting Information B.

3.3. Nuclear protein enrichment confirmed by shotgun mass spectrometry analysis

Protein composition of the isolated nuclei was analyzed by shotgun mass spectrometry. The isolation procedure was conducted twice from two cerebral hemispheres. Fractions of nuclear proteins were purified and analyzed by LC-MS/MS. In effect, 196 and 239 proteins were identified in the samples. Among these, 160 proteins were common in both samples (Supporting Information, Fig. S2). Observed overlap (~70%) was at the same level as overlap observed between two MS analyzes carried out as technical replicates (Elias et al., 2005). This confirms that the samples were prepared in a reproducible manner, what allowed to study merged data. Therefore, a total list of 265 proteins was generated by compilation of the two data sets using the ProteinScape software. The lists of proteins are included in Supporting Information B.

In order to explore cellular localization of the identified proteins *subcellular location* annotations from the UniProt database were analyzed. Distribution of protein localization in both samples and in the compiled list were very similar (detailed data are presented on Table S1 in Supporting Information A). In the same way set of the whole *Mus musculus* proteome from SwissProt database was investigated (16,677 sequences, October 2014). Generally, many proteins are present in multiple localizations and there is a large population of proteins which was assigned both *nucleus* and *cytoplasm* annotation, so we decided to analyze this group separately. As expected, distribution of cellular localization of obtained proteins was significantly different from the *Mus musculus* proteome (Fig. 4). Percentage of proteins with *nucleus* localization (without

cytoplasm) annotation increased 2.4 times with respect to *Mus musculus* proteome while *nucleus* and *cytoplasm* localization increased 2.6 times. Proteins with these annotations comprised 66.4% from proteins identified in the isolated fractions.

According to the literature, high purity organelle fraction can be achieved only for specific organelles such as mitochondria and chloroplasts (Gatto et al., 2010; Li et al., 2005), therefore such a significant increase in the percentage of nuclear proteins obtained in our experiment indicates satisfactory separation of nuclei from others cellular compartments. On the other hand, part of proteins moves through different cellular compartments and may be present in more than one location, so it is difficult to distinguish full-time residents from contaminants (Gatto et al., 2010).

Classifications of identified proteins were also carried out by the DAVID bioinformatics tool on the basis of GO terms in following areas: cellular component, molecular function and biological process. The most strongly represented annotations are presented in Fig. 5. The enrichments in all categories were calculated using the DAVID as well (detailed lists are presented in Supporting Information B). The highest fold enrichment in cellular component was observed in spliceosome (24.5), nuclear periphery (12.4), nuclear matrix (11.3), nucleosome (10.9) and heterochromatin (10.6). All these structures are parts of the nucleus that confirms efficiency of nuclei enrichment process.

The analysis of molecular function revealed an enrichment of proteins involved in: RS domain binding (play role in splicing, fold enrichment 54.0), translation elongation factor activity (12.5), RNA biding (8.5) and ATP-dependent helicase activity (6.0). The following biological processes were overrepresented in examined samples: nuclear mRNA splicing via spliceosome (26.2), mRNA metabolic process (17.2), protein polymerization (12.1) and RNA transport (10.5).

As expected, proteins involved in transcription were enriched, but overrepresentation of proteins related to translation might raise doubt, however some immunological studies proved that translation factors are present in nucleoplasm (Iborra et al., 2001; Lejbkowicz et al., 1992). Furthermore, the presence of proteins in unexpected location may be associated with their moonlighting functions, which are performed among others by eukaryotic translation elongation factor 1α1, which was identified both in our samples and in Shakib's samples from nuclei (Sasikumar et al., 2012; Shakib et al., 2005).

4. Concluding remarks

Several 2DE maps of nuclear proteins have been published, however, verification of procedure of sample preparation protocol and analysis of enrichment in nuclear proteins have not been performed. The current study is the first comprehensive optimization of proteomic analysis of nuclear proteins isolated from mouse brain. Pure fraction of intact nuclei can be achieved by application of homogenizer with appropriate clearance and purification of nuclei using high-density sucrose solution. Nucleic acids proved to be very hard to remove and required application of several treatments. The proposed procedure involves the minimum number of steps, which include nuclei sonication, nuclease digestion of nucleic acids and protein precipitation using acetone. This combination provides high quality separation of proteins by 2DE and successful LC–MS/MS shotgun identification. GO analysis of obtained proteins confirmed enrichment in nuclear proteins, especially those associated with spliceosome, nuclear matrix, nucleosome and heterochromatin. The presented work may contribute to establish a high standardized procedure, which can be used for detailed proteomics analysis of molecular processes occurring in the mouse brain nucleus.

Conflict of interest statement

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jneumeth.2015.12.002.

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