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DIFFERENT PROTEIN PROFILES IN INFERIOR COLLICULUS AND CEREBELLUM: A COMPARATIVE PROTEOMIC STUDY

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Abstract—The characteristic features of individual brain regions are determined by anatomical, physiological, and biochemical properties, which are caused by the nature and amount of proteins expressed. Proteomics is a powerful technology for assessing different protein profiles, comparing hundreds of proteins simultaneously. Here we performed a semi-quantitative proteomic analysis of two prominent brain regions in the male adult rat, the inferior colliculus and the cerebellum. Both play important roles in sensorimotor integration but have distinct anatomical and biochemical features. Soluble proteins of mainly cytoplasmic origin were obtained through subcellular fractionation, separated by two-dimensional gel electrophoresis, and identified by matrix-assisted laser desorption/ionization mass spectrometry. Out of 169 annotated and quantified spots, 40 (24%) displayed significant differences in intensity between the two brain regions. Of those, 21 spots (containing 26 proteins) were more intense in the inferior colliculus and 19 spots (containing 25 proteins) in the cerebellum. The inferior colliculus displayed a higher abundance of proteins involved in vesicular trafficking, such as dynamin-1 and cofilin-1. In the cerebellum, Ca^{2+}-binding proteins (calbindin and calretinin) as well as 14-3-3 proteins were more abundant. Both protein groups play a central role in cellular signaling. Finally, several differences occurred among proteins involved in cellular energy metabolism. Our study presents a proof of principle to demonstrate marked heterogeneity of proteins between two brain samples. The heterogeneity is likely associated with functional differences, warranting further histological and physiological analyses.

Key words: brain proteomics, gel-image analysis, two-dimensional gel electrophoresis, region-specific differences, mass spectrometry.

The mammalian brain, arguably the most complex organ known, consists of a plethora of heterogeneous regions that differ from each other in anatomical, physiological, and biochemical properties (Becker et al., 2006). These properties are derived from both qualitative and quantitative differences in the protein pattern. For assessing differential protein expression, proteomics is a powerful tool. The major difference to previously existing analytical techniques (e.g. immunochemistry) is that proteomics does not analyze proteins individually, but on a highly automated, large-scale level, thus allowing the simultaneous comparison of hundreds of different proteins.

Several neuroproteomics studies have analyzed the brain as a whole or were aimed at identifying differences between health and disease (Cheon et al., 2001; Zheng et al., 2003; Kim et al., 2004), age-related differences (Chen et al., 2003b), or changes caused by traumata (Denslow et al., 2003). Our strategy complements these approaches by comparing specific brain regions from healthy animals. In the present study, we analyzed the expression profiles of proteins in the inferior colliculus (IC) and the cerebellum of male adult rats. The IC is an obligatory relay station in the auditory system and participates in the temporal integration of sound and in the construction of an initial auditory image used for reflexive behavior (Casseday and Covey, 1996; Davis et al., 2003). The cerebellum controls the timing of coordinated, skilled movements of skeletal muscles, integrating inputs from various brain regions as well as the spinal cord (Thach and Bastian, 2004; Ito, 2005). Degeneration or loss of cells in the cerebellum leads to unsteadiness of gait, miscoordination of movement, and ataxia (Gilman, 2000). Thus, both regions are involved in sensorimotor integration, guiding appropriate motor responses, albeit in different context.

Our comparative proteomics study was designed as a proof of principle study to assess whether distinct, but presumably small, differences between two brain regions can be resolved. Combining two-dimensional gel electrophoresis, quantification with specialized software, and matrix-assisted laser desorption/ionization (MALDI) –mass spectrometry (MS) based protein identification, we elucidated a series of proteins with different, i.e. region-specific expression levels. This demonstrates that an analysis of individual brain regions is feasible by gel-based proteomics. It is a useful approach to analyze developmental processes or disease models, where changes may be brain-region specific.

EXPERIMENTAL PROCEDURES

Reagents

Acrylamide and other reagents for polyacrylamide gel electrophoresis, as well as most other chemicals, were obtained from Roth (Karlsruhe, Germany). SB3-10 was supplied by Sigma (Munich, Germany) and the protease inhibitor cocktail by Roche (Mannheim, Germany).

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Abbreviations: AF, abundance factor; IC, inferior colliculus; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; RuBP, Ruthenium II tris bathophenantrolinedisulfonate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Animals and preparation

Young adult male Sprague–Dawley rats (8–9 weeks old) were obtained from Charles River Laboratories (Sulzfeld, Germany) and kept 48 h in our local facilities for acclimatization. Animals were deeply anesthetized by a peritoneal injection of 700 mg/kg chloral hydrate and killed by decapitation. Protocols were approved by the responsible animal care and use committee (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany) and adhered to the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals and their suffering. After removal of the brain from the skull, the cerebellum was separated from the underlying brainstem by cutting horizontally through its pedunculi (Fig. 1a). The cortical hemispheres were separated by cutting along the midline, thus exposing the colliculi (Fig. 1b). The ICs were separated from the superior colliculi and the underlying tegmental brainstem (Fig. 1c). Cerebellum and ICs were prepared conservatively to avoid contamination by surrounding brain regions (Fig. 1d). The tissue samples were collected from single individuals and kept separated for the generation of biological replicates. They were immediately frozen in liquid nitrogen and stored at −80 °C.

Sample preparation

To focus on soluble, predominantly cytoplasmic proteins, the tissue samples were subjected to subcellular fractionation as described previously (Guillemin et al., 2005). In brief, frozen tissue was transferred to lysis buffer (10 mM Hepes, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 5 mM EDTA, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.5), and pre-homogenized by two strokes in a Teflon/glass homogenizer. The suspension was incubated in lysis buffer on ice for 10 min, followed by six strokes of a motorized homogenizer. Thereafter, isotonic conditions were restored with 0.1 volume of 2.5 M sucrose. Differential centrifugation was performed, starting with an initial centrifugation step at 6300×*g* for 10 min to sediment nuclei and cell debris. The supernatant from the first round of centrifugation was subjected to a prolonged centrifugation step (107,000×*g* for 30 min). The resulting supernatant was cleaned up by precipitation with trichloroacetic acid (40%), subsequently washed with acetone (80%), and resuspended in rehydration buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3–10 and 40 mM Tris) prior to two-dimensional gel electrophoresis. Protein concentration was determined by densitometry, comparing a sample aliquot with a dilution series of a protein standard after separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

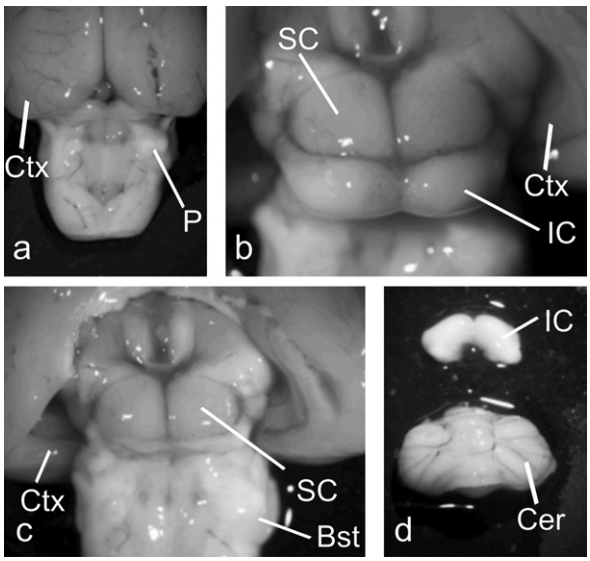

Fig. 1. Preparation of the two rat brain regions. (a) After removing the brain from the skull, the cerebellum was isolated by cutting horizontally through its pedunculi (P). (b) The cortical hemispheres (Ctx) were separated by cutting along the midline, revealing the colliculi below. (c) The IC were separated from the superior colliculi (SC) and the underlying tegmental brainstem (Bst). (d) Cerebellum and IC were prepared conservatively to minimize contamination by surrounding brain regions.

Two-dimensional gel electrophoresis

In order to separate proteins in the first dimension, 18-cm-long immobilized pH-gradient strips (ReadyStrip IPG-strip, pH 3–10, Bio-Rad, Munich, Germany) were used following the manufacturer's instructions. After rehydration (12 h), isoelectric focusing was performed (33,000 Vh) on an IPGphor isoelectric focusing unit (GE Healthcare). After equilibration, the gel strips were transferred to a 12% SDS-PAGE for the second dimension, using a Protean II xi cell (25 mA for 6 h, Bio-Rad).

Both analytical and preparative gels were prepared. Analytical gels were loaded with ~25 μg of protein sample and stained with Ruthenium II tris bathophenantrolinedisulfonate (RuBP), a highly sensitive dye that stains linearly across a large dynamic range (Berggren et al., 2000; Lamanda et al., 2004). Gel images were acquired with a Versadoc 3000 imaging system (Bio-Rad). Preparative gels were loaded with ~500 μg of protein sample and stained with colloidal Coomassie Blue G250 (17% ammonium sulfate, 34% methanol, 0,5% acetic acid, 0,1% Coomassie Blue G250). Gel images were acquired using a gel scanner (GS-800 calibrated densitometer, Bio-Rad).

Protein identification by MS

Protein spots were excised from preparative gels using a spot cutter (ProteomeWorks plus Spot Cutter, Bio-Rad). Excised spots were washed first with 50 mM NH_4HCO_3 and then with 50% acetonitrile/25 mM NH_4HCO_3. Protein disulfides were reduced with 10 mM DTT at 56 °C for 30 min, followed by carbamidomethylation with 5 mM iodoacetamide for 30 min. Finally, the spots were washed again as described above and dried. Proteins were in-gel digested overnight at 37 °C by adding 3 μl of 0.6% (w/v) trypsin (Promega, Madison, MA, USA) in 50 mM NH_4HCO_3. Peptides were extracted by incubating them with 0.1% trifluoroacetic acid for 45 min, then concentrated using PerfectPure C-18 tips (Eppendorf, Hamburg, Germany), and finally eluted onto a MALDI target plate (MTP AnchorChip 384/600, Bruker Daltonics, Bremen, Germany), using α-cyano-4-hydroxycinnamic acid as matrix.

Spectra were acquired using a MALDI-TOF-TOF (time-of-flight) instrument (Ultraflex, Bruker Daltonics) operating in reflector mode with the reflector voltage set to 25 kV. External calibration of the instrument was based on spectra obtained from a mixture of nine standard peptides (Peptide Calibration Standard II, Bruker Daltonics). One-thousand spectra per sample were summed up and processed with FlexAnalysis 2.2 (Bruker Daltonics) to generate a mass list. Internal calibration was achieved using trypsin autoproteolytic fragments present in the spectra. Peptide mass fingerprints were analyzed with Biotools 2.2 (Bruker Daltonics). MASCOT searches were conducted considering carbamidomethylation and oxidation of methionine as variable modifications. Peptide mass tolerance was set at 0.15 Da. Initial searches were performed using the Swissprot database limited to entries for *Rattus norvegicus*. If no significant hits (molecular weight search (Mowse) score of >50; $P>0.05$) were obtained on initial searches, the database size was increased to include all mammalian entries from the NCBI non-redundant database NCBInr. In case of significant hits, matching peptides were removed from the mass list and