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Rat brain proteome in morphine dependence

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Abstract

The aim of this study was to reveal potential markers associated with drug dependence, using the proteomic approach. Gels containing samples derived from morphine-treated and control animals were compared and analyzed. Inspection of protein profiles, following TCA/acetone precipitation and the use of nano-scale liquid chromatography coupled to tandem mass spectrometry, allowed for identification of eleven potential dependence markers, mainly cytoplasmic and mitochondrial enzymes, e.g. proteins that belong to GTPase and GST superfamilies, ATPase, asparaginase or proteasome subunit p27 families.
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1. Introduction

The term ‘‘proteome’’, introduced in 1995 (Wasinger et al., 2001) characterizes the entire phenotype encoded by an organism’s DNA, occurring in a determined space and time. Recently, we introduced the term ‘‘morphinome’’ (Bodzon-Kulakowska et al., 2005) to address protein pattern altered as a result of administration of morphine.

Drug administration can cause addiction and lead to organ damage. Its significant effects have been identified at the molecular, cellular, structural, and functional levels (Sklair-Tavron et al., 1996). Brain subjected to morphine is profoundly different from the non-addicted one, as manifested by changes in its metabolic activity, receptor availability, gene expression and responsiveness to environmental signals (Volkow et al., 2003). For example, morphine induces alterations in biosynthesis and degradation of dopamine (Husi and Grant, 2001). It is believed that the effects of drugs on dopaminergic pathways eventually affect the way the neurons work and communicate in the central nervous system (CNS) (Wasinger et al., 2001). Most drugs that produce elevation of mood or euphoria also cause the release of dopamine in either nucleus accumbens or prefrontal cortex (Nestler et al., 1993). Occasionally, substances of abuse can also affect other neurotransmitters, such as massive release of serotonin by 3,4-methylenedioxymethamphetamine (MDMA) (Kalant, 2001).

It has been shown that opiates generate adaptive changes in intracellular mechanisms (Nestler, 2004; Di Chiara and Imperato, 1988), therefore our goal was to identify possible drug-induced changes that could serve as specific markers of drug dependence.

Such variations, represented by qualitative and quantitative changes in the phenotype, can be monitored by comparison with control tissue (Husi and Grant, 2001). Identification of specific biochemical markers provides an important insight into the processes occurring in the organism during morphine dependence, and can hopefully lead to the ‘‘tailor-made’’ individual pharmacological treatment (Beranova-Giorgianni and Desiderio, 2000; Langen et al., 1999; Fountoulakis et al., 1999; Herbert et al., 2001).

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The first two-dimensional (2D) database of rat brain proteins was constructed by Langen in 1999 (Langen et al., 1999), and since then the knowledge about brain proteins has been updated regularly (Fountoulakis et al., 1999; Lubec et al., 2003; Fountoulakis et al., 2000; Krapfenbauer et al., 2003). However, alterations in the rat brain protein pattern evoked by morphine have not been studied so far, nor specific databases for such brain proteins have been created.

Therefore, with the aid of high-throughput analysis, two-dimensional electrophoresis and nano-scale liquid chromatography coupled to tandem mass spectrometry (nano-LC–MS/MS), we investigated alterations in the expression level of proteins in morphine-treated versus control rat brains.

2. Experimental procedures

2.1. Animals and treatment

Male Wistar rats obtained from a local distributor (HZL, Warsaw, Poland), weighing 150–200 g were used in the experiments. Animals were housed in groups of 5 per cage, in the 12/12 h light/dark cycle in an air-conditioned room. Water and standard food (Bacutil, Motycz, Poland) were freely available.

Morphine dependence was induced by subcutaneous implantation of pellets containing 75 mg of morphine base, according to the modified Way's procedure (Way et al., 1969). Pellets were implanted on the animals' necks under light ether anesthesia. Control animals obtained placebo (0.9% NaCl) instead of morphine. The animals were sacrificed by decapitation 3 days after implantation of pellets. Brains were removed and rapidly frozen on dry ice and stored at −80 °C until analysis.

2.2. Ethical requirements

All experiments were performed in agreement with the respective Polish and European Communities Council Directives (86/609/EEC), and were approved by the Local Ethics Committee (Permission No. 367/2002).

2.3. Materials

Ready-to-use, immobilized pH gradient (IPG) strips, pH 3–10, were purchased from Bio-Rad (USA). Ampholytes, pH 3–10, and *N*-[Tris(hydroxymethyl)methyl]glycine were obtained from Fluka (Steinheim, Germany). Glycine was from POCh (Gliwice, Poland), low protein molecular weight markers were from Amersham Pharmacia Biotech (Uppsala, Sweden). Cholamidopropyldimethylhydroxypropanesulfonate—CHAPS, ammonium persulfate, glycerol, porcine pancreas trypsin treated with L-(tosylamido-2-phenyl)-ethylchloromethylketone (TPCK) came from Sigma–Aldrich (Poznan, Poland). Agarose and CBB R-250 were from Serva (Heidelberg, Germany). Morphine was from Sigma–Aldrich (St. Louis, USA). Other reagents for gel preparation were from Fluka (Steinheim, Germany).

2.4. Preparation of protein samples

The brain hemisphere tissue was suspended in 2 ml of an ice-cold lysis buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 60 mM dithiothreitol (DTT), 2% ampholyte 3–10, 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted in a glass–glass homogenizer and centrifuged at 18,000 × *g* for 1 h at +4 °C. Clear supernatant was divided into two aliquots. One part was frozen immediately, while proteins from the remaining part were precipitated (2 h on ice) with 10% trichloroacetic acid (TCA) in acetone. Subsequently, proteins from TCA/acetone precipitates were solubilized in a solution consisting of 7 M urea, 2 M thiourea, 50 mM DTT, 2% CHAPS, 0.2% ampholytes (pH 3–10) under sonication. Then, the samples were centrifuged (50,000 × *g*, 30 min, +4 °C). The supernatants were divided into small aliquots and stored at −80 °C. Protein content was determined using a modified Bradford method.

2.5. 2D PAGE

Two-dimensional gel electrophoresis was performed. Rat brain proteins from the TCA/acetone precipitates were separated on medium size gels (18.3 cm × 19.3 cm × 0.1 cm). About 2500 μg of each sample was loaded on a 17 cm strip with a pH gradient between 3 and 10. IEF was performed at +20 °C on a PROTEAN IEF Cell (Bio-Rad). Rehydration (16 h) in a buffer containing sample solution was completed in a one-step procedure. Prior to the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)-second dimension, IPG strips were equilibrated for 2 × 10 min by gentle shaking in 3 ml of 0.375 M Tris–HCl, pH 8.8, containing 20% glycerol, 6 M urea and 2% SDS. To the equilibration solution (first equilibration step), 130 mM DTT was added in order to resolubilize the proteins and reduce disulfide bonds. Alkylation was carried out in the dark with 135 mM iodoacetamide (second equilibration step).

PROTEAN II xi Cell from Bio-Rad (USA) was employed for SDS-PAGE electrophoresis, which was carried out using 12% separating gel according to Laemmli buffer system (Laemmli, 1970). Gels were stained with 0.1% Coomassie Brilliant Blue (CBB) R-250 in 40% methanol, supplemented with 10% acetic acid, for about 1 h.

2.6. Pattern evaluation

Gel images were scanned and the data acquired by the white light illuminator, Fluor-S MultiImager. Subsequently, image analyses were performed with the PDQuest 2-D Analysis Software Version 6.2 (Bio-Rad). The process included spot imaging, detection, and placing gels in the match sets for comparison, statistical analysis, and database search. Software system gave position, shape and density information for each of the detected spots. Spot detection and matching were performed simultaneously in a manual manner.

2.7. Sample preparation for mass spectrometry

The spots of interest were excised from the CBB-stained gel with a scalpel, chopped into cubes (ca. 1 mm × 1 mm), rinsed with water and transferred into siliconized centrifuge tubes. CBB stain was removed with 100 μL of 100 mM NH_4 HCO_3 and an equal volume of acetonitrile was added after 10–15 min. For the more efficient stain removal, samples were incubated at +37 °C and vortexed during dye washing. Then, gel pieces were dehydrated with 50 μL of acetonitrile and re-swollen in 13 ng/μL trypsin in 50 mM NH_4 HCO_3 (a volume sufficient to cover the gels) in an ice bucket for 45 min. The supernatant, which was not absorbed by gel particles was removed, and the gel pieces were immersed in 50 mM NH_4 HCO_3 and incubated overnight at +37 °C. After completion of digestion, supernatant was transferred into another tube, followed by the addition of 10 μl 50 mM NH_4 HCO_3, and after 10–15 min an equal volume of acetonitrile was pipetted. The samples were incubated under shaking at +37 °C for 30 min. Extraction of the peptides was repeated twice with 5% formic acid (v/v) in acetonitrile solution (sufficient to cover gel particles). Supernatants were combined, and extracts evaporated to dryness in a vacuum centrifuge.

2.8. Nano-LC–MS/MS

Dried samples were prepared for LC–MS by dissolving in 6 μl 0.1% acetic acid. The LC–MS analysis, used to separate the digests, was performed with the Ultimate LC microchromatography system (LC Packings/Dionex, Amsterdam, The Netherlands). The separation was made on an LC Packings capillary column filled with the PepMap reversed-phase material (15 cm long, 75 μm ID, C18, 2–3 μm bead size and 100 Å pore size).

The gradient was formed using 0.1% HCOOH in water (solvent A) and 0.1% HCOOH in acetonitrile (solvent B), and was delivered at a flow-rate of 200 nL/min. HPLC system was controlled by the Chromeleon software (Dionex). A linear gradient was produced from 1% to 65% B in 30 min. Because of gradient delay caused by the void volumes in the flow-calibration system (ca. 5 μL), the acquisition was prolonged by an additional 20 min. Total analysis time was 60 min, including column equilibration.

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