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Effect of chronic mild stress and imipramine on the proteome of the rat dentate gyrus

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Abstract

The present study uses a proteomic approach to examine possible alterations of protein expression in the hippocampus of rats that are subjected to chronic mild stress (CMS). These rats serve as an animal model that was developed to mimic anhedonia, which is one of the core symptoms of depression. As antidepressant treatment is effective after a few weeks of administration, we also aimed to identify changes that were linked to chronic (once daily for 4 weeks) and 'pulse' (once a week) administration of imipramine. Fifteen differential proteins were identified with 2D electrophoresis followed by mass spectrometry. Although both methods of imipramine administration restored normal sucrose consumption in rats that were subjected to CMS, the molecular mechanisms of these two therapies were different. CMS-induced changes in the levels of dynactin 2, Ash 2, non-neuronal SNAP25 and alpha-enolase were reversed by chronic imipramine, but 'pulse' treatment was not that effective.

Keywords: chronic mild stress, hippocampus, imipramine, proteomics.

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Understanding the biology of depression is a challenging scientific problem with enormous sociological and clinical relevance. The discovery of antidepressant drugs (ADs) and the investigation of their mechanisms of action has revolutionized our understanding of neuronal functioning and the possible mechanisms underlying depression. An emerging hypothesis suggests that the pathogenesis and treatment of depression is likely to involve plasticity of neuronal pathways. Antidepressant treatments may exert their therapeutic effects by stimulating appropriate adaptive changes in neuronal systems (Vaidya and Duman 2001). After decades of effort, the field of depression research is still far from understanding how ADs mediate their clinical effects (Malberg and Blendy 2005).

Both daily dosing of the drug and the elapse of a certain period of time that is necessary for the plastic events to develop are believed to be important for the occurrence of these adaptive changes. The question which of these factors is more important has been addressed in the literature. Although ADs are thought to be effective only after long-term administration, we previously demonstrated that some effects of ADs, which are usually reported in animals after chronic (2–3 weeks) treatment, could be observed also after single administration that followed a 2–3 weeks drug-free period (Kusmider *et al.* 2006). Chiodo and Antelman (1980) were the first to pose that question when they focused on the attenuation of the ability of apomorphine to reduce the firing of the dopaminergic neurons in the rat

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Abbreviations used: ACN, acetonitrile; ADs, antidepressant drugs; BDNF, brain-derived neurotrophic factor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMS, chronic mild stress; DTT, dithiothreitol; IMI, imipramine; MS, mass spectrometry; NIPSNAP1, 4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1; NOS, nitric oxide synthase; NSE, neuron-specific enolase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TNT, Tris-buffered saline with 0.1% Tween-20.

substantia nigra, and they demonstrated that the effect was induced not only by repeated antidepressant treatment but also by a single dose that followed a drug-free period. In line with these findings, we have shown in behavioural studies that the subsensitivity of pre-synaptic dopamine D_2 receptors was observed not only after repeated administration of various ADs, but also after a single dose of these drugs that was followed by two drug-free weeks (Dziedzicka-Wasylewska and Rogoz 1997). Similar effects were observed in behavioural studies at the level of α_2-adrenergic, and dopamine D_3 receptors (Dziedzicka-Wasylewska and Rogoz 1999) as well as at the level of β-adrenergic receptors in binding studies (Kusmider *et al.* 2006). In addition, using the procedure of the repeated FST (forced swim test) – six times over 21 days, we have shown that the shortening of immobility time that is induced by single dose of antidepressants persisted throughout the whole experimental period and was similar to what was seen for the group of animals that were treated repeatedly with the same drug. The mechanism of action for ADs has not been fully understood, despite the well-known pharmacological effects, mainly on monoaminergic pathways, of acute administration of these drugs. There is a temporal mismatch between the rapid alterations in extracellular level of monoamines that are induced by antidepressants and their slow onset of action. This mismatch points to the initiation of adaptive changes, which are responsible for therapeutic efficacy and need time for full manifestation. Antelman elaborated that issue further (Antelman *et al.* 2000) indicating the possibility of time-dependent sensitization which may be responsible for delayed effect of ADs.

As the evidence regarding mechanism of action of ADs is still not strong enough, we decided to use the chronic mild stress (CMS) experimental paradigm to examine the anti-anhedonic effect using two different modes of administering imipramine (IMI). CMS is one of the best animal models of depression (Willner 1997, 2005). It induces anhedonia-like behaviour, which is one of the major symptoms of depression, by exposing the rats to a series of mild stressors for 8 weeks. Such treatment causes, among other deficits, a reduced consumption of, and decreased preference for a palatable sweet solution (Willner *et al.* 1987). This decrease in sensitivity to reward is assumed to model human anhedonia. It has been well established that treatment with different classes of antidepressants during the last 4 weeks of the experiment diminishes anhedonia. In the present experiment, we used a chronic, which is the usual method, administration of IMI to the animals subjected to CMS, and, additionally, a 'pulse' administration of the drug, which was given once a week. In addition to the behavioural measurements that included the consumption of a 1% sucrose solution, we studied the biochemical changes that were induced by stress itself under these two modes of IMI administration.

The main aim of the present study was to investigate the global protein expression differences independent of any hypothesis that described depression aetiology and recovery. We employed proteomics technology, as it allows the investigation of the protein spectrum and its biological functions and, consequently, might lead to a better understanding of changes in previously unexplored proteins. A proteomics analysis comprises two steps, which are the separation of protein mixtures, usually by two-dimensional (2D) gel electrophoresis, and the identification of the separated proteins by various analytical methods, mainly by mass spectrometry (MS) (O'Farrell 1975; Mann *et al.* 1993; Griffin and Aebersold 2001; Aebersold and Mann 2003). The hippocampus was selected as the region of interest for several reasons. One was based upon a previous study that used proteomic investigation in the CMS model (Bisgaard *et al.* 2007). Another was based upon data that pointed to changes in gene expression that were mediated by transcription factors, such as cAMP-response element binding protein upon antidepressant treatment, which is an observation that is best documented in the hippocampus (D'Sa and Duman 2002). In addition, there is a large body of evidence that shows the impact of stress on hippocampal function and plasticity, as demonstrated in electrophysiological, neuroanatomical and cellular physiology studies (Campbell and Macqueen 2004; de Kloet *et al.* 2005). In animal models of depression, the stress-induced suppression of hippocampal neurogenesis has been repeatedly shown to be normalized by chronic treatment with antidepressants (Jayatissa *et al.* 2006, 2008; Surget *et al.* 2008).

The overall aim of the present study was to identify changes that were induced in the rat hippocampus by chronic and 'pulse' treatments with the antidepressant imipramine, IMI. IMI, which is a tertiary amine, is a tricyclic antidepressant of the dibenzazepine group. It affects numerous neurotransmitter systems that are known to be involved in the aetiology of depression (Yildiz *et al.* 2002), and it is one of the ADs that is used most often in animal studies that are aimed at deciphering the mechanisms that underlie the clinical efficacy of antidepressants.

Experimental procedures

Materials and methods

Ready-to-use, immobilized pH gradient strips, pH 3–10 nonlinear gradient, IPG (carrier ampholyte mixture) buffer and DALT Gel 12.5 (255 × 196 × 1 mm) were purchased from GE Healthcare Life Sciences (Uppsala, Sweden). CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), urea, thiourea, bromophenol blue, DTT (dithiothreitol), EDTA, SDS (sodium dodecyl sulphate), trichloroacetic acid, glycine, agarose, sodium acetate, silver nitrate, calcium carbonate, sodium thiosulfate, iodoacetamide, Tris were purchased from Lab Empire (Rzeszow, Poland). Diethanolamine and phenylmethylsulphonyl fluoride were purchased from Merck (Darmstadt, Germany). Glycerol, TFA (trifluoroacetic acid), aceto-

nitrile (ACN), formic acid, acetic acid, formalin, ethanol were purchased from POCh (Gliwice, Poland). Sequencing Grade Modified Trypsin was purchased from Promega (Madison, WI, USA). Imipramine was purchased from Pliva (Cracow, Poland). Rabbit anti-Dynamitin (dynactin 2) polyclonal antibody, rabbit anti-4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (NIPSNAP1) polyclonal antibody, rabbit anti-Synapsin II polyclonal antibody and goat anti-Rabbit secondary antibody were from Abcam (Cambridge, UK). Mouse anti-β-actin monoclonal antibody and goat anti-Mouse secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals and treatment

Animals

The experiments were carried out on male Wistar rats. Water and standard food were freely available, and the animals were kept at a constant room temperature (22°C) under a 12 h light/dark cycle.

Sucrose consumption test

Male Wistar rats that were trained to consume a 1% sucrose solution in a 1-h test that was performed once a week were subjected to a procedure of sequential exposures to a variety of mild stressors for 8 weeks. A decrease in the consumption of the solution was a measure of anhedonia-like state of the rats, which is one of the core symptoms of depression, in the stressed animals.

Experimental data were analyzed with two-way ANOVA for repeated measurements. Analysis has shown no interaction between time and treatment: $F(20,165) = 1.22$, $p = 0.24$. Impact of the treatment factor on measured parameter has been considered significant: $F(4,165) = 9.56$, $p < 0.0001$, as well as the time factor: $F(5,165) = 17.63$, $p < 0.0001$. Bonferroni post-tests indicated the following: stressed animals, during whole period of the experiment, drunk significantly less of the sucrose solution than not-stressed rats. The effect of imipramine administered repeatedly, once a day 10 mg/kg i.p. became significant in 5th week of the experiment. The same effect was observed in both groups receiving the drug in 'pulse' manner, i.e. 10 mg/kg i.p. once a week, on a day before or a day after the sucrose intake test. Contemporary the mean values of sucrose intake for all three groups at that time point did not differ significantly, although were slightly smaller, from the mean value of not-stressed animals.

Chronic mild stress protocol

On the basis of their sucrose intakes in the final three baseline tests, the animals were divided into two matched groups and placed in separate rooms. One group was exposed to an initial 2 weeks of chronic mild stressors, and the other was left undisturbed. The stress protocol consisted of one period of intermittent illumination, stroboscopic light, grouping and food or water deprivation, two periods of a soiled cage with no stress, and three periods of 45° box tilting. During these periods, the rats were housed in pairs, alternating between the partner being the normal resident or an intruder. All of the stressors lasted from 10 to 14 h.

After 2 weeks of stress exposure, which is when the sucrose intake between the stressed and control animals started to differ significantly, the stressed animals were further divided into four groups. The first subgroup was treated with vehicle, the second was treated with chronic (i.e. once daily) IMI 10 mg/kg i.p., the third was treated with IMI 10 mg/kg i.p. once a week on a day before the sucrose test, and the fourth was treated with IMI 10 mg/kg i.p. once a week on a day after the sucrose test. The last two groups were undergoing 'pulse' therapy, in order to determine whether or not the presence of IMI in the animals will influence the sucrose intake. In addition, there was the control group of animals, which were not subjected to CMS. Each group consisted 8–10 animals.

As there was no difference in sucrose intake between the two 'pulse' treated groups, only one of them (imipramine after test) was included in the biochemical part of experiment. Therefore, the analysis of the hippocampal proteome was performed in four groups, which were the control, the CMS-subjected, the CMS-subjected treated chronically with IMI and the CMS-subjected treated with 'pulse' therapy.

Tissue processing

After the experiment animals were killed by decapitation, the brains were rapidly dissected, and the hippocampi were removed. For proteomic analysis of the hippocampus, fragments containing the dentate gyrus was punched out form the frozen slices (bregma −2.80 to −4.16 mm; cryostat CM 3000, Leica/Jung, Germany, at −21°C). Tissue fragments were stored at −80°C until analysis. The results presented in this study are obtained from three to five animals per group.

Ethical requirements

The experiments were performed in accordance with the regional legal regulations.

Preparation of protein samples

The hippocampus tissue was suspended in ice-cold buffer that contained 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris pH 8.5, 0.002% bromophenol blue, 65 mM DTT, 1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride and homogenized with a homogenizer (Miccra D-1, Carl Roth GmbH, Karlsruhe, Germany) on ice. After homogenization (for approximately 3 × 10 s), the suspension was left at 4°C for 1.5 h and centrifuged at 14 000 *g* for 60 min at 12°C. The supernatant was precipitated with 10% trichloroacetic acid and then incubated for 1.5 h at 4°C. After that, the suspension was centrifuged at 10 000 *g* for 30 min at 12°C. The remaining pellet was rinsed with cold acetone for 10 min and then centrifuged at 10 000 *g* for 5 min at 4°C. The precipitated proteins were solubilized in a rehydration buffer that was composed of 7 M urea, 2 M thiourea, 2% CHAPS, 0.002% bromophenol blue, 0.5% IPG buffer and 20 mM DTT (the IPG buffer and DTT were added just before using). The protein concentration in samples was determined by Bradford assay (Bradford 1976).

Two-dimensional electrophoresis

Samples that contained 200 µg of total protein were applied to 24 cm strips with an immobilized pH 3–10 nonlinear gradient. IEF was performed at 20°C on the Ettan IPGphor 3 IEF System (GE Healthcare, Uppsala, Sweden). Rehydration (12 h) was completed in a one-step procedure. Focusing started at 500 V for 1 h, voltages of 1000 V were applied for 1 h, voltages of 8000 V – for 1 h and voltages of 10 000 V for 10 h. Prior to SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) – second dimension, the IPG (immobilized pH gradient)

strips were equilibrated for 2 × 10 min by gentle shaking in a 75 mM Tris–HCl pH 8.8 buffer that contained 30% glycerol, 6 M urea and 2% SDS. One, 1% DTT was added to the buffer in the first equilibration step. Alkylation was carried out in the dark with 2.5% iodoacetamide for the second equilibration step.

After the first dimension separation, the proteins were separated on large size gels (255 × 296 × 1 × mm, 12.5% polyacrylamide). SDS–PAGE was performed on the Ettan DALTsix Large Vertical System (GE Healthcare) at 2 W/gel for 45 min and 16 W/gel for 4.5–5 h at 20°C.

After protein fixation for 12 h in 25% ethanol and 10% acetic acid, the gels were stained by silver nitrate according to Yan (Yan *et al.* 2000), with modifications. Images were acquired with ImageScanner III (GE Healthcare, Uppsala, Sweden) and analyzed by ImageMaster 2D Platinum v6.0 (GE Healthcare, Uppsala, Sweden). The image analysis software was used for spot detection, quantification and analysis according to the manufacturer's instructions. Briefly, the analysis scheme consisted of a few steps: detection of spots, identification of landmark proteins, aligning and matching of spots in gels, quantification of matched spots and manual inspection of the spots to verify the accuracy of matching. Spot detection settings (smooth – 3, minimum area – 50, saliency – 50) were adjusted so that a maximum of visible spots were detected. The spot volume was used as the analysis parameter for quantifying protein expression. The protein spot volume was normalized to the spot volume of all the protein spots – % Vol. Intensity normalization was additionally used to compensate for sample loading and staining variations.

Protein identification by mass spectrometry

Differential spots were cut from the 2D gels; the same spots from particular group were pooled and suspended in 10% acetic acid. Many of differential spots found exhibited small intensity, so pooling procedure increased the chance of correct protein identification by MS. Peptide mixtures were analyzed by liquid chromatography coupled to an LTQ FT ICR mass spectrometer (Hybrid-2D-Linear Quadrupole Ion Trap – Fourier Transform Ion Cyclotron Resonance Mass Spectrometer, Thermo Electron Corp., Bremen, Germany). Prior to the analysis, gel slices were subjected to a standard 'in-gel digestion' procedure, during which proteins were reduced with 100 mM DTT for 30 min at 56°C, alkylated with iodoacetamide for 45 min in a darkroom at 22°C and digested overnight with sequencing Grade Modified Trypsin. The resulting peptides were eluted from the gel with 0.1% TFA and 2% ACN. The peptide mixture was applied to an RP-18 pre-column (LC Packings) using water that contained 0.1% TFA as the mobile phase and than transferred to a nano-HPLC RP-18 column (nanoACQUITY UPLC BEHC18, Waters) using an acetonitrile gradient (0–60% ACN in 30 min) in the presence of 0.05% formic acid with a flow rate of 150 nL/min The column outlet was directly coupled to an ion source of LTQ-FT-MS that worked in the regime of data dependent MS to MS/MS switch. A blank run that ensured a lack of cross-contamination from previous samples preceded each analysis.

Acquired raw data were processed by Mascot Distiller (version 2.1.1, Matrix Science, London, UK) followed by Mascot Search database search engine (v.2.1, Matrix Science), locally installed http://matrixscience.com) against the NCBI or SWISS-PROT non-redundant database with Rodentia as taxonomic category to allow protein identification. Protein mass was unrestricted. Search parameters for precursor and product ions mass tolerance were respectively ± 60 ppm and ± 0.8 Da, with an allowance made for one missed semiTrypsin, fixed modifications of cysteine through carbamidomethylation and variable modifications through lysine carbamidomethylation and methionine oxidation. Only proteins containing three or more unique peptides with an individual Mascot cut-off score > 42 indicating identity or extensive homology ($p < 0.05$) were taken into further consideration.

Western blotting

Samples containing 7.5 μg of protein for dynactin 2 and NIPSNAP1 or 19.5 μg for synapsin II from rat hippocampus were resolved by 12% SDS–PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked in Tris buffered saline containing 5% non-fat milk powder overnight at 4°C. Blocked membranes were incubated for 2 h with anti-Dynamitin rabbit polyclonal antibody (dilution 1 : 1000), anti-Synapsin II rabbit polyclonal antibody (dilution 1 : 8000) in Tris-buffered saline with 0.1% Tween-20 (TNT). After four TNT buffer washes, anti-Rabbit IgG with horseradish peroxidase-conjugated (dilution 1 : 8000) was applied to the membranes for 1 h at 22°C. The membranes were then washed with TNT buffer and subjected to enhanced-chemiluminescence reaction (ECL, Santa Cruz Biotechnology) before exposure to X-ray films. The same blot was re-probed for β-actin which served as loading control using anti-β-actin mouse monoclonal antibody (dilution 1 : 1000 – 1 : 2000) and anti-Mouse IgG with horseradish peroxidase-conjugated (dilution 1 : 5000). The intensity of each band on the western blot was quantified with Bio-Rad Quantity One software (Hercules, CA, USA).

Results

Sucrose consumption

The consumption of the 1% sucrose solution in the control group was stable over the whole period of the experiment (Fig. 1). In animals that were subjected to the CMS procedure, the sucrose consumption was much lower, which demonstrated the anhedonic effect of stress. Animals receiving IMI treatment every day started to drink the more palatable solution, and the effect was significant on the fifth week of the experiment. The same effect was observed in the group of animals that were treated with the same dose of IMI (10 mg/kg) at a frequency of once a week instead of once a day.

Proteomic analysis

Protein extracts from four groups of rats were separated by 2D electrophoresis, and the proteins spots were visualized by silver nitrate staining. In each group, samples from at least three animals were analyzed. Protein sample from each individual was studied on a separate gel.

Representative examples of the hippocampus proteins from four groups, separated in a 2D gel, are shown in Fig. S1(a–d). Spot detection parameters were set as follows:

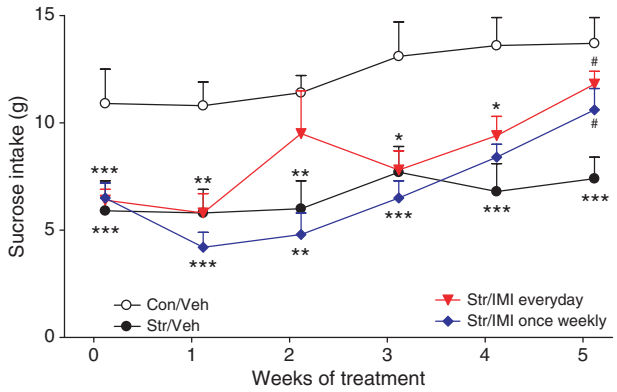

Fig. 1 Sucrose consumption. Effects of chronic treatment with vehicle (1 mL/kg), IMI (10 mg/kg) once daily or IMI (10 mg/kg) once weekly on the consumption of a 1% sucrose solution in controls (open symbols) and in animals exposed to chronic mild stress (closed symbols). Treatment commenced following 2 weeks of stress. Values are mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; relative to vehicle-treated controls. #$p < 0.05$; relative to drug-treated stressed animals at Week 0.

smooth 3, minimal area 50, saliency 50. The number of spots that were found in the control group was 2520 ± 482, in the stressed group was 2480 ± 244, in the group that was treated chronically with IMI (once a day) was 2691 ± 349 and in the group that was treated with IMI once a week ('pulse') was 2789 ± 134. The average percent matches* between the masters of groups was 65%.

In order to make the analysis easier, five spots (which were highly reproducible in all gels of four groups and well separated from others spots) were cut and analyzed by MS. The identified proteins served in further analyses as 'standard' proteins (Table 1, Fig. S1a–d). The 'standard proteins' were used to create approximate grid line of molecular weight and pH value. It was necessary because of nonlinear pH gradient was applied.

Because of the limitation of staining methods, the analysis was focused on qualitative differences in proteins level. The only quantitative differences analysed were those that have been found when spots in compared groups differed in a percent of volume by at least a factor of 1.5 (Wu *et al.* 2009). Statistically significant quantitative changes in protein expression were determined using p value of ≤ 0.05 as analysis criteria.

As a result, 45 spot differences were identified (Fig. 2). The isoelectric points (pI) and masses of the spots were determined with a computer program on the basis of the pI values and molecular weights (MWs) of the five marker proteins.

*Percent matches was calculated as follows: $2m/(n_t + n_c)$, where m is the total number of matches between the master of control group and master of treated group, n_t is the number of spots in the master of treated group and n_c is the number of spots in the master of control group.

Table 1 'Standard' spots measured by MS

Spot	pI	MW [kDa]	Swiss-Prot	Protein name
A	7.98	15.9	P02091	Hemoglobin subunit beta-1
B	4.41	17.4	P35434	S-phase kinase-associated protein 1A
C	5.95	62.6	P47942	Dihydropyrimidinase-related protein 2
D	6.67	28.8	P25113	Phosphoglycerate mutase 1
E	5.21	25.5	Q00981	Ubiquitin carboxyl-terminal hydrolase isozyme

The 'standard' proteins served in further analysis as marker proteins to determine the pI value and MW of identified proteins with the computer program.

Fifteen proteins were identified by MS on the basis of peptide mass matching following digestion with trypsin, and their peptide sequences were obtained in a secondary ionization process. The peptide masses and sequences were matched with the theoretical peptide masses by using the Mascot program (http://www.matrixscience.com) and a non-redundant The National Center for Biotechnology Information (NCBI) database for rodents. Remaining spots probably contain not enough protein to be faithfully detected and identified by MS analysis even despite the pooling of cut samples which belong to the same group.

The NCBI accession numbers, the abbreviated and full names of the proteins, their theoretical pI and molecular weight values, their functions, and data from the mass spectrometry analysis, such as their score and the protein amino acid sequence coverage by the matching peptides are presented in Table 2.

When comparing the control and CMS groups, twelve proteins had detectable changes in their levels (Table 2, original numbers 1–12). The following proteins had increased levels upon stress: dynactin 2, Ash-m, F-box protein 2, calmodulin, annexin A3, galactokinase 1, aldolase C, fructose-biphosphate. The levels of five of the proteins were decreased, and these were NIPSNAP1, alpha enolase, synapsin IIb, poly C binding protein, malate dehydrogenase 1, NAD (soluble).

In the case of four proteins, which were dynactin 2, Ash-m, 4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1, and alpha enolase, chronic administration of IMI reversed the effect of CMS (Table 2, original numbers 1–4). However, 'pulse' administration of IMI either did not reverse the effect of CMS or had a much lower effect when compared with chronic administration.

Three proteins, which included dynamin 1, pyruvate dehydrogenase E1 alpha 1, and glyceraldehyde-3-phosphate dehydrogenase, had no change in their levels upon stress, but

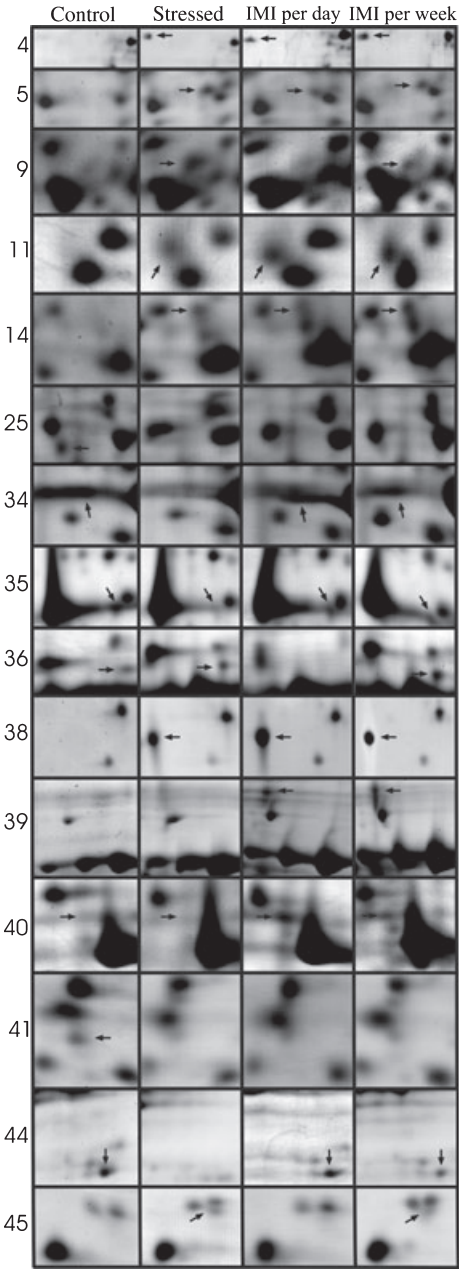

Fig. 2 The zoomed changes in the level of spots identified by MS in four studied group of animals: control, stressed control, imipramine (IMI) once per day, imipramine (IMI) once per week. Numbers on the left indicate the spot number as listed in Table 2.

chronic administration of IMI influenced their levels (Table 2, original numbers 13–15).

The level of three interesting proteins: dynactin 2 and synapsin was examined by western blot and the results are presented in Fig. 3. The obtained results are in reasonable agreement with the data from 2D electrophoresis. Because of higher sensitivity of western blot, the spots invisible on 2DE gel were detectable in western blot experiments.

Discussion

As has been shown in a number of papers (see Introduction), some behavioral and biochemical alterations, described commonly after repeated administration of ADs to experimental animals can be detected also after single dose of the drug, however following at least several drug-free days. As it has been widely assumed that CMS experimental paradigm is one of the best animal models mimicking some symptoms of human depression, in the present studies we decided to test our working hypothesis that it is the time rather than the daily dosing of the drug necessary to trigger the adaptive changes that lead to the altered functioning of an organism (Kusmider *et al.* 2006).

The results that were obtained from the behavioral experiment indicate that IMI restores normal sucrose consumption in the rats subjected to the CMS procedure, and there was no difference between drug administration in the conventional, chronic way, and drug administration in the 'pulse' manner (i.e. once a week). Such an effect is very interesting and indeed indicates that 'pulse' dosage of the drug is sufficient to induce the 'antidepressive effect'. That result by no means questions the validity of the model – rather it stresses for the need of re-evaluation of our understanding of the mechanism of action of ADs. The clinical data supporting this finding are scarce as yet (Pollock *et al.* 1986, 1989; Jagadeesh *et al.* 1992; Dube *et al.* 1996; Malhotra and Santosh 1996; Deuschle *et al.* 1997; Sallee *et al.* 1997; Antelman and Gershon 1998), however it is understandable at present – first there must be more evidence provided by pre-clinical studies to convince physicians for using the pulse rather than everyday treatment to their patients. In our opinion such an approach, if successful, would definitely diminish the unwanted side effects, induced by daily drug dosage. However, we are perfectly aware that such an approach has to be carefully designed and based on more pre-clinical studies.

On the other hand, the search for biochemical changes that underlie the observed effects was not fully successful, as chronic treatment with IMI did not induce the same alterations as the 'pulse' regimen at the proteome level. Basically 'pulse' treatment caused lower effect than chronic drug administration.

The combination of 2D electrophoresis and mass spectrometry has resulted in the identification of fifteen proteins that are differentially expressed in rat hippocampus after chronic mild stress and treatment with the AD, imipramine.

The proteins that were found to be modulated by the CMS procedure are involved in several different cellular processes. Dynactin 2 and calmodulin, which were both up-regulated and synapsin IIb, which was down-regulated, are involved in neuronal vesicular cell trafficking and synaptic plasticity. Ash-m and annexin A3, which were both up-regulated, are involved in signalling. 4-nitrophenylphosphatase domain and

Table 2 Differentially expressed proteins in rat hippocampus after CMS, chronic and 'pulse' treatment with imipramine

		Average value % VOL						Mascot results			Experimental		From sequence	
#	Spot #	Control	Stress	IMI once a day	IMI once a week	NCBI #	Protein name	Score	Peptides matched[a]	Seq. coverage %	pI	MW	pI	MW
1	9	0.0000	0.0178	0.0000	0.0103	gil51948450	Dynactin 2	437	7	23	4.97	49 904	5.14	44 235
2	45	0.0000	0.0142	0.0000	0.0130	gil914957	Ash-m	204	3	22	5.79	24 037	6.31	23 655
3	44	0.0456	0.0000	0.0179	0.0113	gil6679066	4-Nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1	96	3	10	9.92	29 399	9.48	33 570
4	34	0.0615	0.0000	0.0639	0.0404	gil38649320	Alpha enolase (enzyme)	480	9	31	6.08	47 793	6.70	51 736
5	4	0.0000	0.0187	0.0149	0.0272	gil16758270	F-box protein 2	258	5	22	4.29	38 840	4.30	34 021
6	38	0.0000	0.2858	0.3988	0.2254	gil28948873	chain A, Crystal Structure Of Endothelial Nitric Oxide Synthase Peptide Bound To Calmodulin which is identical with calmodulin sequence	699	3	49	4.19	16 275	4.09	16 515
7	11	0.0000	0.0183	0.0177	0.0114	gil6978503	Annexin A3	190	6	20	5.94	33 025	6.04	36 528
8	5	0.0000	0.0142	0.0107	0.0110	gil56605662	Galactokinase 1 (enzyme)	297	4	14	4.92	41 245	5.24	42 806
9	14	0.0000	0.0251	0.0186	0.0280	gil6978489	Aldolase C, fructose-bisphosphate (enzyme)	318	5	14	6.68	40 627	6.67	39 658
10	25	0.0174	0.0000	0.0000	0.0000	gil112350	Synapsin IIb – rat	273	4	11	6.28	59 477	7.62	52 822
11	41	0.0181	0.0000	0.0000	0.0000	gil495128	Poly C binding protein	143	3	14	6.21	39 144	8.48	35 336
12	35	0.0809	0.0395	0.0431	0.0471	gil15100179	Malate dehydrogenase 1, NAD (soluble) (enzyme)	266	5	22	6.15	34 445	6.16	36 631
13	39	0.0000	0.0000	0.0171	0.0469	gil18093102	Dynamin 1	547	11	17	7.30	81 878	6.32	96 209
14	40	0.0104	0.0167	0.0288	0.0173	gil6679261	Pyruvate dehydrogenase E1 alpha 1 (enzyme)	422	9	30	7.14	44 267	8.49	43 888
15	36	0.0192	0.0130	0.0000	0.0171	gil8393418	Glyceraldehyde-3-phosphate dehydrogenase (enzyme)	168	4	15	7.89	35 633	8.14	36 090

[a]Total number of peptides assigned to the protein. Multiple matches to the peptides with the same primary sequence was counted as one.

The first section of the table represents proteins which responded to CMS and chronic imipramine treatment inversed the effect of CMS.

The second section of the table depicts proteins which responded to CMS but chronic imipramine treatment did not further influence the levels of these proteins.

The third section of the table represents proteins which did not respond to CMS but their levels changed upon chronic drug administration.

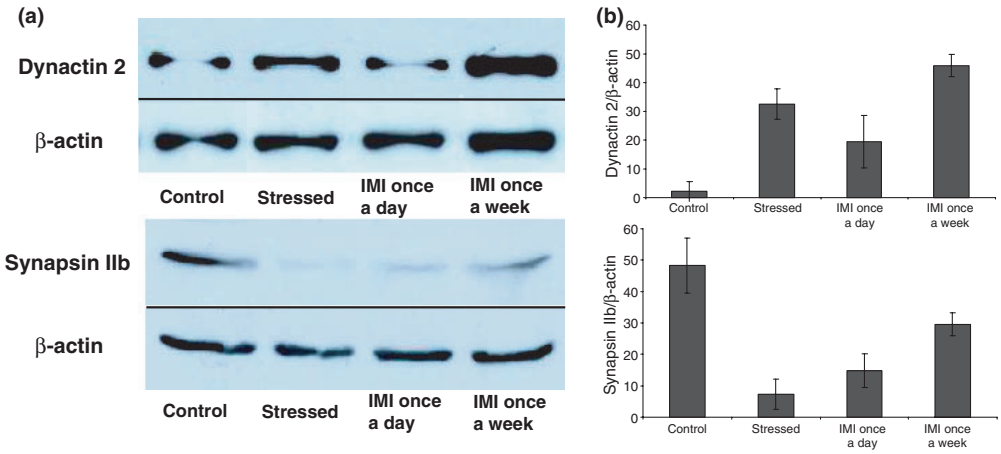

Fig. 3 (a) Western blot analysis of dynactin 2 and synapsin IIb expression in rat hippocampus in four investigated groups: control, control stressed, chronic Imipramine administration (IMI once a day), 'pulse' Imipramine administration (IMI once a week). The expression of β-actin is depicted below as an internal control. The PVDF membrane was blotted or re-blotted separately for each target protein. (b) The intensity of each band obtained by western blotting was quantified by Bio-Rad Quantity One software. The data columns represent particular proteins expression as relative levels per β-actin. The experiment was performed at least three times. Data are mean ± SD.

non-neuronal SNAP25-like protein homolog 1, which was down-regulated, is involved in the regulation of transcription. Poly C binding protein, which was down-regulated, is involved in the regulation of transcription and translation. The F-box protein, which was up-regulated, is involved in protein degradation processes. Four of these proteins also appeared to be simultaneously modulated by IMI, and these were dynactin 2, Ash-m, 4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1, and alpha enolase. For these cases, daily administration of IMI reversed the effect of stress.

Dynactin 2 is required for bidirectional organelle transport, and its presence is necessary for the development of the axon growth cone (Abe *et al.* 1997; Deacon *et al.* 2003). Dynactin plays a role in intracellular and axonal transport, and disturbance of its function could impact important cellular processes within various compartments of the motor neurons. This protein is up-regulated in animals that are subjected to CMS, which might indicate an intensive process of synapse formation. This could serve as a mechanism for compensating for the stressful conditions. Chronic treatment with IMI decreased its level to that in the untreated, control group. 'Pulse therapy' with IMI, however, was less efficient in the reduction of dynactin 2 levels.

Ash-m is physically associated with dynamin. It plays a role in the signalling pathway between the epidermal growth factor receptor like tyrosine kinase and the Ras GDP/GTP exchange protein, which allows the ligand-activated receptor tyrosine kinase to modulate Ras activity (Watanabe *et al.* 1995). Dynamin is a GTPase that is required for synaptic vesicle endocytosis. Although dynamin levels were not altered in the CMS group of rats and did not have any detectable levels in the control group, it was up-regulated by IMI treatment. Dynamin binds to the Ash SH3 domain through its C-terminal proline-rich region, and this, in turn, stimulates GTPase activity. In addition to dynamin, two other brain-specific proteins that bind to the SH3 domain of the Ash protein in rat brain were found, which were synapsin I and p145. Synapsin I is a synaptic vesicle-associated nerve terminal protein that interacts with actin and is thought to mediate the interactions of synaptic vesicles with the presynaptic cytomatrix. p145 is present exclusively in neurons and co-localizes with dynamin, which suggests an important role in endocytosis (McPherson *et al.* 1994). In the bovine brain p150 and p65 were found to be Ash-binding proteins, and both seemed to be expressed exclusively in the brain and localized to nerve terminals. Therefore, Ash may function as a regulator of synaptic vesicle transport either directly or indirectly through dynamin and other proteins (Miura *et al.* 1996). Here, we showed that CMS caused an increase in Ash-m levels and that chronic IMI reversed this effect.

Another interesting protein, which showed changes upon the CMS procedure and response to the IMI treatment, is NIPSNAP1. It is a ubiquitously expressed protein (Satoh *et al.* 2002), and the function of NIPSNAP1 remains unknown. NIPSNAP1 probably plays an important role in signal transduction or regulation of the cytoskeleton in the post-synaptic density (Surendran *et al.* 2005). CMS down-regulated the level of this protein, and repeated treatment with IMI reversed this effect, restoring it to control levels. However, 'pulse' dosage of the drug was not able to do the same.

The level of neuron-specific enolase (NSE) was changed in the same manner as NIPSNAP1. NSE is an enzyme of the glycolytic pathway, and it is found in numerous isomeric forms. Alpha enolase and gamma enolase are present

specifically in neuronal cell cytoplasm and dendrites. Beyond serving as a neuronal marker, NSE can be released from distressed neurons into the cerebrospinal fluid and peripheral blood, which allows it to also serve as a biomarker of parenchymal brain injury (Kulig 1991). Moreover, alpha enolase was found to be up-regulated in human brain disease (Castegna *et al.* 2003), up-regulated in mouse models of Alzheimer's disease (Tilleman *et al.* 2002), and down-regulated in a rat model of schizophrenia (Paulson *et al.* 2003). Interestingly, we found that administration of IMI reversed the effect of stress on alpha enolase. Results that were obtained after chronic treatment with another antidepressant, GR205171, which is an mammalian tachykinin receptors 1 (NK1) tachykinin receptor antagonist, also showed changes in alpha enolase (Carboni *et al.* 2006a).

Among the proteins that were detected in the present study, there were some that were altered by the CMS procedure, where treatment with IMI was not able to reverse these changes. Within this group of proteins, F-Box protein 2, calmodulin, annexin, and galactokinase 1 were all up-regulated by CMS, and synapsin IIb, pyruvate dehydrogenase, poly C binding protein, and malate dehydrogenase were all down-regulated.

The F-box is a protein motif of approximately 50 amino acids that functions as a site of protein-protein interactions (Kipreos and Pagano 2000), and it participates in protein degradation and controls the life cycle of cells. Interestingly, aberrations in the ubiquitin pathway have been linked to neurological conditions (Hagens *et al.* 2006). In our proteomic analysis this protein is not apparent in the control group; it starts to be detectable in all groups of animals that were subjected to CMS, and IMI treatment does not alter this change. As the high level of F-box protein 2 has been linked to excessive protein degradation and the atrophy of the hippocampus, this finding corresponds well with the data that shows that a lower hippocampal volume is associated with depression (Frodl *et al.* 2006).

Among proteins identified by Mascot searching there is the chain A of the crystal structure of endothelial nitric oxide synthase peptide bound to calmodulin but detailed analysis of its sequence indicated that the really found protein is calmodulin. In present studies the level of calmodulin was increased upon stress. Similar result was earlier observed by Carboni in proteomic studies of the influence of repeated psychosocial stress on rat hippocampus (Carboni *et al.* 2006b). Calmodulin and calmodulin-regulated proteins are engaged in nerve growth process and they are suggested to modulate the synaptic plasticity that is induced by stress in the hippocampus (Kim and Yoon 1998). The enzyme nitric oxide synthase (NOS) is exquisitely regulated *in vivo* by calmodulin to control the production of NO, which is a key signalling molecule and cytotoxin. Recent studies showed that NO regulates the expression of brain-derived neurotrophic factor (BDNF), which affects neural progenitor cell proliferation and differentiation in the mammalian brain (Canossa *et al.* 2002; Cheng *et al.* 2003). BDNF exerts a neuroprotective effect via down-regulation of neuronal NOS activity (Sharma *et al.* 1998), but it was also shown that endothelial NOS-deficient mice have decreased BDNF expression in the ischemic brain (Aoyagi *et al.* 2003; Chen *et al.* 2005). In contrast, Canossa *et al.* (2002) reported that a non-specific NOS inhibitor increased BDNF secretion, and a NO donor decreased BDNF secretion in studies with cultured embryonic hippocampal neurons. The molecular mechanism that underlies endothelial NOS regulation of neurogenesis is still unknown.

Annexin A3 is another protein that is found to be up-regulated by CMS and not sensitive to IMI treatment. It is a signal protein in the hippocampus, and its level changes with development of this structure (Chen *et al.* 2007; Weitzdörfer *et al.* 2008). On 2D electrophoresis images, the annexins are present in two different spots (Chen *et al.* 2007).

On the other hand, synapsin II b and poly C binding protein were detected only in the control group of animals and down-regulated in all the groups that were subjected to CMS. This observation is interesting, as synapsin II b regulates the reserve pool of synaptic vesicles and is required for maintaining vesicles in the reserve pool at excitatory synapses but its role is still not so clear. It has been postulated that synapsin could be involved in the development of neurons (Bogen *et al.* 2006). According to Villanueva *et al.* (Villanueva *et al.* 2006) deletion of the synapsin gene caused an increase in the secretion of neurotransmitters to the synaptic cleft. The pI value was determined to be 6.30 in this study (Table 2), and it differs from the isoelectric point that was determined based on the amino acid sequence, which was 7.62. This observation suggests that the examined spot contained the phosphorylated protein. The presence of synapsin in the control group indicates that there is no disturbance in catecholamine secretion. The lack of this protein in the stressed groups can, in turn, be interpreted to be evidence for a perturbation in neurotransmitter secretion. However, IMI did not abolish this perturbation, although it does inhibit the reuptake of catecholamines. On the other hand, the down-regulation of synapsin by ADs was also described by Carboni *et al.* (2006a).

Similarly, the poly C binding protein is also down-regulated in groups that were subjected to CMS, whether or not these groups also received IMI. This protein belongs to the group of poly C binding proteins. The roles of these proteins in mRNA stabilization, translational activation, and translational silencing suggest a complex and diverse set of post-transcriptional control pathways. Their additional putative functions are connected to transcriptional control and structural components of important DNA-protein complexes (Makeyev and Liebhaber 2002). As a transcription regulator, poly C binding protein is involved in the regulation of mu-opioid receptor gene expression (Kim *et al.* 2005). The

results that were obtained in the present study indicate that the CMS procedure has a strong influence on these pathways.

Among these identified proteins, there are four enzymes with altered expression levels that changed in response to CMS. Malate dehydrogense, which was two-fold down-regulated, mentioned above alpha enolase, which was down-regulated, and fructose biphosphate aldolase C, which was up-regulated, were found earlier by Carboni in a similar animal model of depression as being down-, up-, and up-regulated, respectively (Carboni *et al.* 2006b). Fructose biphosphate aldolase C was found earlier to be up-regulated in schizophrenia, bipolar disorder, and depression in studies that were carried out on postmortem human brains (Johnston-Wilson *et al.* 2000).

Galactokinase is also an enzyme that is up-regulated under stress conditions, although it is not sensitive to IMI treatment. It is known that galactose as well as its derivatives, such as galactose-1-phosphate, regulates the activity of the Na^+, K^+-ATPase in rat brain (Tsakiris *et al.* 2002). Therefore, it may modulate the neural excitability, metabolic energy production, catecholaminergic system, and serotoninergic system. It has been shown that galactose-1-phosphate causes the inhibition of Na^+, K^+-ATPase activity in whole brain homogenates.

The fact that a lot of proteins that were identified in this proteomic analysis were enzymes suggested that altered oxidation metabolism may be involved in the development of the depression-like state. This hypothesis has been suggested by Mu in a summary of proteomic analyses that described the influence of a rat depression model on hippocampal proteins (Mu *et al.* 2007). Metabolic pathways in neurodegenerative and psychiatric disorders are usually deranged, and many proteins that have altered levels and modifications are involved in energy metabolism pathways. These proteins are mainly high-abundance enzymes (Fountoulakis and Kossida 2006). Similar results were obtained by Johnston-Wilson *et al.* (Johnston-Wilson *et al.* 2000), in their postmortem studies of protein expression in human frontal cortex of patients who were suffering from psychiatric disorders. Six of the eight identified proteins that were determined to be important in psychiatric disorders were enzymes.

In addition, we noticed that the levels of two other enzymes were changed after imipramine administration in the CMS treated animals. Pyruvate dehydrogenase, which was 1.8-fold up-regulated, was also identified by Khawaja *et al.* (2004) in human frontal cortex that was examined by 2D electrophoresis as being up-regulated after chronic treatment with the ADs, fluoxetine, which is a selective serotonin reuptake inhibitor, and venlafaxine, which is a dual serotonin/norepinephrine reuptake inhibitor. This enzyme is engaged in anti-apoptotic events, so it can prevent the lowering of hippocampus volume, which is associated with depression. The pI value for pyruvate dehydrogenase, after it was taken from spot localization on the 2D electrophoresis gel, was determined to be 6.30, but the isoelectric point that was based on the amino acid sequence was equal to 7.62. This difference suggested that the examined spot contained the phosphorylated protein. This type of modification for this enzyme was found in earlier studies of the synapotosomal protein by shotgun LC-MS/MS analysis (Witzmann *et al.* 2005). Glyceraldehyde-3-phosphate dehydrogenase, which was down-regulated after IMI treatment, belongs to a group of multiple protein expression forms that represent splicing forms or co- and post-translational modifications. This is similar to the previously noted malate dehydrogenase, which is involved in tricarboxylic acid cycle, and fructose biphosphate aldolase C, which is involved in glycolysis (Yang *et al.* 2005). Glyceraldehyde-3-phosphate dehydrogenase is an enzyme that is involved in the glycolysis process and its level, which was in opposition to the results from the present work, increased in response to chronic treatment with IMI, when detected at the mRNA level from amygdala tissue samples (Tohda *et al.* 1999). Glyceraldehyde-3-phosphate dehydrogenase, at both the mRNA and protein levels, also increased in response to treatment with other antidepressants (Drigues *et al.* 2003; Carboni *et al.* 2006a). However, the fact that different tissues were used in these studies may account for that discrepancy.

An interesting result observed in the present study concerns the cytoskeleton protein, dynamin 1. IMI administration induces the up-regulation of this protein. As mentioned above, that change correlates with the down-regulation of Ash-m levels, and both these proteins directly cooperate. It has been also shown that, similarly to IMI, another compound with antidepressant activity, GR205171, which is an mammalian tachykinin receptors 1 (NK1) tachykinin receptor antagonist, caused an increase in the dynamin 1 level when administered chronically (Carboni *et al.* 2006a).

In conclusion, the proteins that were defined as markers in the present study are potentially important factors that are involved in the mechanism of chronic mild stress and antidepressant action. Identification of these proteins may allow a better definition of the signal pathways that are important for, or at least involved in, the recovery from CMS following IMI treatment. On the other hand, these results indicate that, although the administration of IMI in a chronic way (once a day) and in a 'pulse' way (once a week) results in the same behavioral effects, the molecular mechanisms behind these two 'therapies' seem to be different. Based upon these results, it appears that further experiments are necessary to elucidate which changes in the proteins profile are essential for antidepressant action.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1. Silver-stained 2D electrophoresis maps of proteins from rat hippocampal extracts.

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