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Proteomic and metabolomic profiling reveals time-dependent changes in hippocampal metabolism upon paroxetine treatment and biomarker candidates

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ABSTRACT

Most of the commonly used antidepressants block monoamine reuptake transporters to enhance serotonergic or noradrenergic neurotransmission. Effects besides or downstream of monoamine reuptake inhibition are poorly understood and yet presumably important for the drugs' mode of action. In the present study we aimed at identifying hippocampal cellular pathway alterations in DBA/2 mice using paroxetine as a representative *Selective Serotonin Reuptake Inhibitor* (SSRI). Furthermore we identified biomarker candidates for the assessment of antidepressant treatment effects in plasma. Hippocampal protein levels were compared between chronic paroxetine- and vehicle-treated animals using *in vivo* ^{15}N metabolic labeling combined with mass spectrometry. We also studied the time course of metabolite level changes in hippocampus and plasma using a targeted polar metabolomics profiling platform. *In silico* pathway analyses revealed profound alterations related to hippocampal energy metabolism. Glycolytic metabolite levels acutely increased while Krebs cycle metabolite levels decreased upon chronic treatment. Changes in energy metabolism were influenced by altered glycogen metabolism rather than by altered glycolytic or Krebs cycle enzyme levels. Increased energy levels were reflected by an increased ATP/ADP ratio and by increased ratios of high-to-low energy purines and pyrimidines. In the course of our analyses we also identified myo-inositol as a biomarker candidate for the assessment of antidepressant treatment effects in the periphery. This study defines the cellular response to paroxetine treatment at the proteome and metabolome levels in the hippocampus of DBA/2 mice and suggests novel SSRI modes of action that warrant consideration in antidepressant development efforts.

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1. Introduction

Major depression is one of the leading causes of morbidity and mortality worldwide. Antidepressant treatment which selectively inhibits monoamine reuptake alleviates symptoms of depression only after several weeks of medication in only a subset of patients (Berton and Nestler, 2006). Novel antidepressants with rapid onset and high treatment efficacy are in great demand. Biomarkers that facilitate prediction of treatment outcome are required in this endeavor.

Besides monoamine reuptake inhibition other downstream effects have been implicated in alleviating symptoms of depression (Moretti et al., 2003). An in-depth analysis of currently used antidepressants at the cellular and molecular level may reveal novel targets for drug development. Direct pharmacological targeting of relevant cellular pathways represents a promising strategy for the development of novel antidepressants. Non-hypothesis driven approaches – like transcriptomics, proteomics or metabolomics – can identify such cellular pathway alterations (Filiou et al., 2011) and represent suitable tools to investigate antidepressant treatment effects (Sillaber et al., 2008).

Abbreviations: FDR, False Discovery Rate; GABA, γ-Aminobutyric acid; MeOH, methanol; mg, milligram; min, minute; ml, milliliter; *m/z*, mass-to-charge ratio; PLS-DA, Partial Least Squares Discriminat Analysis; R, Pearson's correlation coefficient; RP, Reversed Phase; s, second; SAM, Significance Analysis of Microarrays; SRM, Selected Reaction Monitoring; SSRI, Selective Serotonin Reuptake Inhibitor; VIP, Variable Importance in Projection; w/v, weight/volume.

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With the help of unbiased metabolite profiling we have recently shown that chronic paroxetine treatment targets hippocampal glucose metabolism and identified antidepressant drug target candidates (Webhofer et al., 2011).

In the present study we have extended our efforts to identify cellular alterations at the proteome level after chronic paroxetine treatment using ^{15}N metabolic labeling combined with mass spectrometry. To corroborate our findings we investigated cellular metabolism by using a targeted polar metabolomics profiling platform (Yuan et al., 2012). In order to shed light on the delayed onset of therapeutic SSRI action in patients we performed time course experiments and studied acute versus chronic treatment effects on metabolic pathways. Furthermore, we have identified a biomarker candidate that can be used for monitoring hippocampal antidepressant treatment effects in the periphery.

2. Materials and methods

2.1. Paroxetine treatment and organ sampling

Male DBA/2OlaHsd mice (8 weeks old at arrival, Harlan Winkelmann, Borchen, Germany) were singly housed upon arrival for two weeks under standard laboratory conditions (food and water *ad libitum*, 12 h dark–light cycle with lights on at 7 am, 45–55% humidity, 21 ± 2 °C).

After habituation mice were treated twice per day (between 8 and 9 am and 6–7 pm) with paroxetine (10 mg/kg, paroxetine-hemihydrate, Sigma–Aldrich, St. Louis, MO, USA) or vehicle (tap water) by gavaging. Paroxetine was prepared freshly before each treatment by dissolving it in tap water (1 mg/ml). One hour after the last treatment mice were euthanized by an overdose of isoflurane (Forene®, Abbott, Wiesbaden, Germany). Blood was drawn by heart puncture and collected in EDTA tubes (Kabe Labortechnik, Nuembrecht-Elsenroth, Germany). Plasma was separated from serum by centrifugation (1300g, 10 min, 4 °C). Organs were perfused with 0.9% ice-cold saline solution (Merck, Darmstadt, Germany). Mice were decapitated, brains harvested and dissected. Plasma and hippocampi were shock frozen in liquid nitrogen and stored at −80 °C until further analysis.

The experiments were performed in accordance with European Communities Council Directive 86/609/EEC. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

2.2. Quantitative proteomics analysis

2.2.1. Protein sample preparation

Hippocampal soluble proteins were extracted according to Emili and Cox (Cox and Emili, 2006). To all biological replicates the same ^{15}N-labeled internal standard was mixed at equal protein amounts. *In vivo* ^{15}N-labeled hippocampal proteins were derived from DBA/2 mice that were raised with a ^{15}N mouse diet (Silantes GmbH, Munich, Germany) for 12 weeks (Filiou et al., 2011). One hundred μg of the $^{14}N/^{15}N$ protein mixture were separated by one-dimensional SDS gel electrophoresis. Separated proteins were fixed and stained with Coomassie Brilliant Blue R-250 (Biorad, Hercules, CA, USA). The gel was destained and each gel lane was cut into 2.5 mm slices (22–23 slices per biological replicate) and tryptic in-gel digestion and peptide extraction were performed as described previously (Filiou et al., 2011).

2.2.2. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Tryptic peptides were dissolved in 0.1% formic acid and analyzed with a nanoflow HPLC-2D system (Eksigent, Dublin, CA, USA) coupled online to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples were on-line desalted for 10 min with 0.1% formic acid at 3 μl/min (Zorbax-C18 (5 μm) guard column, 300 μm × 5 mm, Agilent Technologies, Santa Clara, CA, USA) and separated via RP-C18 (3 μm) chromatography (in-house packed Pico-frit column, 75 μm × 15 cm, New Objective, Woburn, MA, USA). Peptides were eluted with a gradient of 95% acetonitrile/0.1% HCOOH from 10% to 45% over 93 min at a flow rate of 200 nl/min. Column effluents were directly infused into the mass spectrometer via a nanoelectrospray ion source (Thermo Fisher Scientific). The mass spectrometer was operated in positive mode applying a data-dependent scan switch between MS and MS/MS acquisition. Full scans were recorded in the Orbitrap mass analyzer (profile mode, m/z 380–1600, resolution $R = 60000$ at m/z 400). The MS/MS analyses of the five most intense peptide ions for each scan were recorded in the LTQ mass analyzer in centroid mode. Other MS parameters were set as described previously (Filiou et al., 2011).

2.2.3. Protein identification and quantitation

Peptides were identified by ^{14}N and ^{15}N database searches against a decoy Uniprot mouse protein database (release 2010_02) containing 119,128 entries (including forward and reverse sequences) using Sequest (implemented in Bioworks v3.3, Thermo Fisher). Enzyme specificity was set to trypsin. Mass accuracy settings were 10 ppm and 1 Da for MS and MS/MS, respectively. Two missed cleavages were allowed, carboxyamidomethylation of cysteine was set as fixed and oxidation of methionine as variable modifications. ^{15}N peptide identification was facilitated by a variable modification of −0.9970 Da for lysine and arginine to account for the frequent shift from ^{15}N monoisotopic to most intense ^{15}N isotopomer (at 90% ^{15}N incorporation) as described previously (Zhang et al., 2009). Peptide hits were filtered at a 'False Discovery Rate (FDR)' of 1% using PeptideProphet, ^{14}N and ^{15}N database searches were combined using iProphet and protein groups were detected using ProteinProphet using default parameters (FDR < 1%)(Keller and Shteynberg, 2011).

Relative protein quantitation was performed with the ProRata software (v1.0) using default parameters and excluding ambiguous peptides (Pan et al., 2006). $\log_2$ ($^{14}N/^{15}N$) ratios of protein groups were then compared between biological replicates. Briefly, protein identifications were assigned to the protein group's $\log_2$ ($^{14}N/^{15}N$) ratio. As protein identifications from different biological replicates could be assigned to distinct protein groups (due to the existence of different peptide sets identified by shotgun proteomics) we initially considered each protein identification individually for inter-experimental comparison. To avoid redundancies in final protein quantification, protein identifications with identical $\log_2$ ($^{14}N/^{15}N$) ratios assigned to the same protein group were combined again.

2.3. Targeted metabolomics analysis

2.3.1. Polar metabolite extraction

Hippocampal tissue was ground (2 min × 1200 min^{-1}, homogenizer PotterS, Sartorius, Göttingen, Germany) in 30-fold (w/v) ice-cold 80% MeOH. Samples were centrifuged (14,000 g, 10 min, 4 °C) and supernatants incubated on dry ice. Tissue pellets were further disrupted in 6-fold (w/v) ice-cold 80% MeOH (Branson Sonifier, Branson Ultrasonics, Danbury, CT, USA) and combined with previous supernatants. Plasma metabolites were extracted twice in 80% ice-cold MeOH. Metabolite extracts were vortexed and centrifuged (14,000 g, 10 min, 4 °C). Supernatants were evaporated and stored at −80 °C until further analysis.

2.3.2. Targeted LC-MS/MS

Samples were re-suspended using 20 μl LC-MS grade water for mass spectrometry. Ten μl were injected and analyzed using

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