Hippocampal proteomic and metabonomic abnormalities in neurotransmission and oxidative stress pathways in a chronic phencyclidine rat model

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Introduction: 525 words
Material and Methods: 2262 words
Results: 949 words
Discussion: 1481 words
Abstract

Schizophrenia is a neuropsychiatric disorder affecting 1% of the world’s population. Due to a broad range of symptoms and wide heterogeneity, current therapeutic approaches in schizophrenia fail to treat all symptomatic manifestations of the disease. Therefore, new models that reproduce core pathological features of schizophrenia are needed for the elucidation of unidentified pathological disease mechanisms. Here, we employ a comprehensive global label-free liquid chromatography-mass spectrometry (LC-MS) proteomic and metabonomic profiling analysis combined with targeted proteomics (selected reaction monitoring and multiplex-immunoassay) of serum and brain tissues from a chronic phencyclidine (PCP) rat model, in which NMDA-receptor hypofunction is induced through non-competitive N-methyl-D-aspartate (NMDA) antagonism. Using multiplex immunoassay, we identified serum abnormalities in cytokines (IL-5, IL-2, IL-1β) and the fibroblast-growth factor-2 levels, as peripheral biomarkers of PCP treatment. In order to find potential novel drug targets and to elucidate the pathways associated with the disease, extensive proteomic and metabonomic brain tissue profiling was employed. This revealed a more prominent effect of chronic PCP treatment on the hippocampal proteome and metabonome compared to the frontal cortex. Bioinformatic pathway analysis confirmed the abnormalities in NMDA-receptor associated pathways in both brain regions, as well as alterations in other neurotransmitter systems such as kainate, AMPA and GABAergic signalling in the hippocampus. Metabonomic profiling revealed changes in phospho- and glycolipids, which was further supported by findings of oxidative stress (superoxide dismutase) on the protein level. These molecular changes parallel findings observed in human schizophrenia. The present study could lead to increased understanding of how perturbed glutamate receptor signalling affects other relevant biological pathways in schizophrenia and therefore support drug discovery efforts for improved treatment of patients suffering from this debilitating psychiatric disorder.
Introduction

The potent N-methyl-D-aspartate (NMDA) antagonist phencyclidine (PCP) is of high interest in the study of psychiatric disorders. PCP is a psychotomimetic (positive symptoms) agent,\(^1,\,2\) which is also able to induce negative\(^1,\,3\) and cognitive\(^4\)-\(^8\) symptoms of schizophrenia and other psychiatric disorders, in humans and rodents.\(^9,\,10\) In addition, it can exacerbate these symptoms in schizophrenia patients.\(^11\) Thus, studies of PCP feature prominently in pharmaceutical drug discovery efforts since most current treatments of schizophrenia are predominately tailored against the positive symptoms, while negative and cognitive symptoms tend to be resistant to current antipsychotics. Mechanistically, PCP shows an affinity to a variety of receptors [sigma, dopamine D(2) and 5-HT(2) receptors\(^12,\,13\)] but principally acts at the NMDA receptor (NMDAR), where it binds with high affinity to a specific site within the pore of the ion channel, leading to a NMDAR hypofunction. However, PCP is also a potential antagonist for the dopamine (D2) receptor, inducing abnormalities of dopaminergic transmission across different brain regions in primates\(^14,\,15\) and rodents.\(^16\)-\(^19\) Thereby, PCP models both glutamate and dopamine dysfunction, which represent the key neurotransmitter systems implicated in the pathophysiology of schizophrenia.\(^20\) However, other neurotransmitter systems, such as serotonin/norepinephrine,\(^16,\,19,\,21,\,22\) gamma-aminobutyric acid (GABA),\(^20,\,23\) acetylcholine\(^24\) and opioid system pathways are also affected by PCP treatment, albeit to a lesser extent.\(^25\)

Rodent PCP models are currently among the most commonly used in schizophrenia drug discovery. Previous studies have characterized these models mainly at the behavioural level, with the finding of shared behavioural abnormalities of hyperlocomotion, stereotypy,\(^26\) decreased voluntary sucrose consumption,\(^27\) impaired information processing with cognitive functions of memory\(^28\) and attention and impaired social interaction.\(^26,\,29\)-\(^31\) The behavioural readouts are thought to correspond to the positive, negative and cognitive symptoms as seen in human schizophrenia. Fewer studies have been performed at the physiological and molecular level, although these have implicated abnormalities of neurotransmitter release and levels,\(^16,\,28,\,32\)-\(^35\) dendritic branching, and dendritic spine number, synaptic loss,\(^36\) along with hypofrontality.\(^37\) To date, the molecular mechanisms by which PCP exerts diverse neurochemical, behavioural and clinical effects are still a matter of debate. The first proteomic studies of an acute PCP rat model revealed abnormalities in protein phosphorylation patterns in the frontal cortex\(^38\) and energy metabolism and signal transduction in the hippocampus.\(^39\) Less has been done to investigate the chronic PCP effects in the brain, although it has been noted that repeated use of PCP by humans induces a more persistent schizophrenic symptomatology, including psychosis, hallucinations, delusions, formal thought disorder and cognitive dysfunction and social withdrawal.\(^1,\,40\)-\(^42\) One extensive proteomic and metabonomic study
which investigated the molecular effects in the frontal cortex of a chronic PCP rat model, only led to the identification of subtle abnormalities in proteins involved in calcium signalling and energy metabolism.43

Here, we attempt to gain further insights into the effects of chronic PCP treatment of rats using a combination of metabonomic and proteomic profiling of rat brain tissue. Since only subtle molecular changes have been found in the frontal cortex from chronic PCP treated rats, this study now analyses hippocampal tissue from cPCP treated rats to investigate how this brain region is affected, particularly considering its role in cognition and memory, social behaviour and the (negative) symptom domains. The molecular characterisation will help to understand pathophysiological mechanisms in psychosis and schizophrenia and may facilitate future drug discovery and development by evaluating the translational validity of the model.

Material and Methods

Animals

Adult male Sprague–Dawley rats (200–300g; Charles River, Margate, UK) were housed in groups of four under standard laboratory conditions with food (Harlan UK, Bicester, UK) and water available ad libitum. All experiments were conducted during the light cycle and were in full compliance with the Home Office Guidance (UK Animals Scientific Procedures Act 1986) and ethical policies of the Home Office. After a 10-day adaptation period, rats were given a daily subcutaneous dose of vehicle (0.9% sterile saline) or PCP HCl (5mg/kg) for 15 consecutive days. All animals were killed by decapitation 30min after the last injection and frontal cortices were dissected on ice and frozen immediately in liquid nitrogen. Behavioural readouts were recorded as described and PCP injection induced the standard increase in locomotor activity and stereotypic movement.43

Serum profiling

Serum samples were analyzed using the RodentMAP, Rat MetabolicMAP and Rat KidneyMAP platforms comprising multiplexed immunoassays of 89 analytes in a Clinical Laboratory Improved Amendments (CLIA)-certified laboratory at Myriad-RBM (Austin, TX, USA) as described previously. Immunoassays were calibrated using duplicate standard curves for each analyte and raw intensity measurements converted to protein concentrations using proprietary software. Multiplexed calibrators (eight levels per analyte) and controls (three levels per analyte) were used to monitor key performance parameters, such as lower limit of quantification, precision, cross-reactivity, linearity, spike-recovery, dynamic range, matrix interference, freeze-thaw stability and short-term sample
stability (http://www.myriadrbm.com/technology/data-quality/). Data analyses were performed using the statistical software package R (http://www.r-project.org) and the levels of analytes were determined. Analyses were conducted under blinded conditions with respect to sample identities and samples were analyzed in random order to avoid any sequential biases. For data analysis, all missing values, zeros and negative values were replaced by the half of the minimum positive value, assuming this to be the detection limit. Analytes with more than 30% missing values were removed. 66 analytes remained for relative quantification (Table S1). Furthermore, approximately 10% of the data were filtered out based on relative standard deviation (RSD). Row-wise normalization to each median reading was employed to adjust for differences among samples and data were log-transformed and pareto-scaled (mean-centered and divided by the square root of standard deviation of each variable) to make features more comparable. Significance Analysis of Microarray (SAM) was performed using the Siggenes R package. SAM is a well-established statistical method for identification of differentially expressed genes in microarray data analysis and is frequently employed for analysis of high-throughput Omics-datasets. It is designed to address the false discovery rate (FDR) when running multiple tests and high-dimensional data. SAM assigns a significance score to each variable based on change relative to the standard deviation of repeated measurements. For a variable with scores greater than an adjustable threshold, its relative difference is compared to the distribution estimated by random permutation of the class labels. For each threshold, a certain proportion of the variables in the permutation set will be identified as significantly different by chance. This proportion is used to calculate the FDR.

Metabonomics

Metabonomic Sample Preparation

Metabonomic profiling was performed as described previously. In brief, approximately 10mg frontal cortex and 30-50mg hippocampus tissues were weighed out into 2mL bead beater tubes and homogenized with 1.45mL of pre-chilled methanol/water (1:1) and 100 μL of 1-mm zirconium beads, using a Precellys bead beater. Homogenisation (6,5000Hz speed) cycles were 40s, followed by cooling on dry ice, and a further 40s homogenisation and cooling on dry ice. The mixtures were then centrifuged at 10,000g for 10min at 4°C. Supernatants (aqueous extracts) were collected and transferred to clean Eppendorf tubes. Aqueous extracts were dried in a vacuum concentrator (Savant) for at least 180min at 45°C. Extracts were resuspended in 120μL of methanol/water (1:1), followed by brief vortexing and sonication, and transferred into 96-well plates for analysis. Quality control (QC) samples were prepared by combining an aliquot (10μL) from each study sample to
produce a representative sample – this was used for column conditioning and data quality assessment as described by Want et al.\textsuperscript{46}

**UPLC-MS analysis of frontal cortex and hippocampus tissues**

UPLC-MS analysis was performed using a Waters XEVO G2 Q-TOF mass spectrometer coupled online to an Acquity UPLC-MS system (Waters Corporation, Milford, MA). Separation was performed at 0.4mL/min and 50°C, using a 2.1 x 100mm (1.7μm) HSS T3 Acquity column. The injection volume was 5μL and the sample temperature was 4°C. The mobile phases were 0.1% (vol/vol) formic acid in water (A) and 0.1% (vol/vol) formic acid in methanol (B). The gradient was (99.9% A for 2 min, to 75% A in 4 min; to 20% A in 6 min, to 10% A in 2 min, to 0.1% A in 7 min, 23 min, 0.1% A for 2 min, to 99.9%A in 4 min). Acquisition was performed in both positive ion mode (1.0kV ESI +) and negative ion mode (1.0kV ESI-). Source conditions were: source temperature: 120°C, desolvation temperature: 350°C, cone gas flow: 25 L/h, desolvation gas flow: 900 L/h. QC samples were injected ten times at the start of the analytical batch in order to condition the column, then after every ten samples throughout the run to assess instrument stability.

Data were processed using the freeware XCMS\textsuperscript{47, 48} using standard parameters. The output consisted of a matrix of metabolite feature m/z, retention time and intensity (peak area) values. These “metabolite feature” tables were imported into SIMCA-P for multivariate analysis (e.g. PCA) to check data quality and sample outliers. All missing and zero values (0.02% of the data) were replaced by the half of the minimum positive value found within the data. The assumption of this approach is that most of the missing values are caused by low abundance metabolites. Since zero values may cause problems for data normalization (i.e. log), they were replaced with this value. Data were filtered to identify and remove variables that were unlikely to be of use when modelling the data. 10% of data points showing little variance across experimental conditions were filtered based on relative standard deviation.\textsuperscript{44, 49} This filtering procedure is highly recommended for chemometric data, which often contains a large amount of noise. Row-wise normalization to sample median was employed to adjust for differences among samples and data was log transformed and pareto-scaled (mean-centered and divided by the square root of standard deviation of each variable) to make features more comparable. P-Values were determined using Wilcoxon’s signed-rank test and corrected to control for multiple hypothesis testing.\textsuperscript{50} Ratios were calculated for each molecule as the mean intensity values of cPCP-treated rats divided by those of controls.
Proteomics

Proteomic Sample Preparation

Tissue samples were added to fractionation buffer containing 7M urea, 2M thiourea, 4% CHAPS, 2% ASB14, 70mM dithiotreitol (DTT) and protease inhibitor at a 5:1 (v/w) ratio. Samples were sonicated (10s, 2 cycles) and vortexed at 4°C for 30 min. Samples were then centrifuged at 17,000 g at 4°C. Protein concentrations of the lysates were determined using a Bradford assay (Bio-Rad; Hemel Hempstead, UK). Approximately 100 μg sample was precipitated using acetone. After dissolving the precipitate in 50mM ammonium bicarbonate, reduction of sulfhydryl groups were performed with 5mM DTT at 60°C for 30min and alkylation was carried out using 10mM iodoacetamide at 37°C for 30 min in the dark and proteins were subsequently digested using trypsin at a 1:50 (w/v) ratio for 17h at 37°C. Reactions were stopped by addition of 8.8 M HCl in a 1:60 (w/w) ratio. Quality control (QC) samples were prepared to monitor machine and preparation performance. For this, an equal amount of each sample was pooled into one sample after the sonication step and then split into multiple aliquots. Each QC sample underwent all experimental steps in parallel with the test samples.

Label-free LC–MS² analysis of frontal cortex and hippocampus tissue

Individual brain tissue samples were analyzed in duplicates using a Splitless nano-ultra-performance liquid chromatography (UPLC) (10 kpsi nanoAcquity; Waters Corporation, Milford, MA) coupled online through a New Objective nanoESI emitter (7 cm length, 10-mm tip; New Objective, Woburn, MA) to a Waters Q-TOF Premier mass spectrometer. Data were acquired in expression mode (MS²) and the total continuous run time was 8 days. The procedure, quality assessment and data processing were performed as described previously.³⁹ LC-MS² data were processed using the ProteinLynx Global Server (PLGS) v.2.4. (Waters Corporation) and Rosetta Elucidator v.3.3 (Rosetta Biosoftware, Seattle, WA) was used for time and mass/charge alignment of mass spectrometry data. The Swiss-Prot human reference proteome (Uniprot release March 2013, 20 252 entries) was used for protein identification searches. Only peptides detected in both replicates and in >80% of samples were included in further analyses. Protein abundance changes were determined using the MSstats².15 package⁶² based on linear mixed-effects models, following log₂ transformation and exclusion of values which deviated by more than 3 standard deviations from the mean of each group. Data were quantile normalized. The p-values were adjusted to control for the false discovery rate (FDR) at a cut-off of 0.05 following the Benjamini-Hochberg procedure.⁵⁰

Protein Pathway analysis
Protein set enrichment analysis

Significantly changed proteins were partitioned into three bins, according to their ratio: ratio<1.0; ratio >1.0 and ratio both >1, <1. Entrez gene rat identifiers were translated to the correspondent identifier in mouse to increase the GO-term coverage. The R package database org.mouse.eg.db version 2.8.0 was used for gene ontology (GO) term annotation based on entrez gene identifiers. Significant over-representation of an annotated GO term in each bin was determined by the GOstats package.\textsuperscript{53} P-values for the GO category\textsuperscript{54} “biological pathway” (BP) were calculated by a conditional hypergeometric test, using the entire detected proteome as a background. These tests accounted for the hierarchical structure of the GO terms by first testing the “child terms” of any given GO category and filtering significantly enriched proteins prior to analysis of the “parents terms”, as described previously.\textsuperscript{55} This prevented the identification of directly-related GO terms with a considerable overlap of assigned proteins. GO terms with no significant enrichment in any bin (p>0.05) and GO terms with less than two annotated proteins were removed. The remaining p-values (>0.05) were replaced by a conservative p-value of 1. P-values were log\textsubscript{10}-transformed and converted to z-scores within their proteomic comparison for every remaining GO term. Finally one-way hierarchical clustering using “Euclidean distance” as distance function and the “Average Linkage Clustering” method available in the Genesis software\textsuperscript{56}, was performed on all significantly enriched GO terms.

Label-based selected reaction monitoring (SRM) mass spectrometry

Abundance alterations of a panel of 18 candidate proteins implicated in NMDAR function were measured using targeted SRM mass spectrometry on a Xevo TQ-S mass spectrometer (Waters Corporation; Milford, CT, USA) coupled online through a New Objective nanoESI emitter (7 cm length, 10-mm tip; New Objective) to a nanoAcquity UPLC system (Waters Corporation). The system was comprised of a C18 trapping column (180µmx20mm, 5µm particle size) and a C18 BEH nano-column (75µmx200mm, 1.7mm particle size). The buffers used for separation were (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile and the following 48 min gradient was applied: 97/3% (A/B) to 60/40% in 30 min; 60/40% to 15/85% in 2 min; 5 min at 15/85%; returning to the initial condition in 1 min. The flow rate was 0.3µL/min and the column temperature was 35°C. In-house multiplex SRM assays were developed using a high-throughput strategy\textsuperscript{57} (see Figure 1a) and the initial process assessed the suitability of over 200 selected proteins. Therefore, initially up to 12 unique peptides ranging from 6 to 20 amino acids in length, containing tryptic ends and no missed cleavages were chosen for each of the selected proteins. All peptides containing amino acids prone to undergo modifications (e.g., Met, Trp, Asn and Gln), potential ragged ends, or those with
lysine/arginine followed by proline or bearing NXT/NXS glycosylation motifs were avoided and only selected when no other options were available. Peptides were also checked by Protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches to ensure uniqueness. Subsequently, up to 12 transitions per were tested in SRM mode for each of the 12 selected peptides on quality control samples. Transitions were calculated using Skyline version 1.2.0.3425 and corresponded to singly charged y-ions from doubly or triply charged precursors, in the range of 350-1250 Da. Transitions were selected based on software internal predictions, discovery proteomics data and spectral data available through the Human NIST spectral libraries.

For the final SRM assay, the 2-3 peptides with the maximal intensities and highest spectral library similarity (dotp) were selected. We also analysed heavy-label spiked QC samples (Figure 1b) in scheduled SRM mode to confirm peptide identities via co-elution, extracted the optimal fragment ions for SRM analysis, obtained accurate peptide retention times and optimized collision energy and cone voltage for quantification runs applying the Skyline software (MacCoss Lab Software; Seattle, WA, USA). Heavy labelled forms of the selected peptides (spiketides L) were chemically synthesized via SPOT synthesis (JPT Peptide Technologies GmBH, Berlin, Germany). The final transitions, collision energies and retention time windows used for each peptide can be found in the supplementary information (Supplementary Table S2a and S2b).

Quantitative SRM measurements comparing chronic PCP treated rats and controls were performed in scheduled SRM acquisition mode, using the optimized parameters defined during assay refinement. For each target peptide, a heavy isotope labelled internal standard (JPT Peptide Technologies GmbH) was spiked in the peptide mixture for accurate quantification and identification. All SRM functions had a 2 min window of the predicted retention time and scan times were 20ms. For each peptide, at least three transitions were monitored for the heavy and light versions. Samples were run randomized and blocked in triplicates, and blanks and quality control peptide injections (yeast alcohol dehydrogenase; Supplementary Table S2c) were run alternating after each biological replicate. Resulting SRM data were analyzed using Skyline and statistical analysis was conducted using SRMstats.

Data pre-processing consisted of a log$_2$ transformation to stabilise the variance. Quantile normalization was performed based on reference transitions to equalize the median peak intensities of reference transitions from all proteins across all MS runs and adjust the bias to both reference and endogenous signals. Protein level quantification and testing for differential abundance among chronic PCP treated rats and control animals were carried out using the linear mixed-effects model implemented in the R-package MSstats 2.15, which employs a “restricted” scope of conclusions. In the restricted scope model, the individual samples being modelled are the population of interest.
This approach also took into account the measurement error of transitions across runs (technical variation), to enable accurate quantification of protein abundance changes across the samples. The $p$-values were adjusted to control the false discovery rate at a cut-off of 0.05 according to Benjamini and Hochberg.\textsuperscript{50}
Results

Serum Profiling

We measured serum levels of 89 analytes in rats following cPCP or vehicle treatment using multiplex immunoassays. This was carried out to elucidate how the effects of PCP are manifested in the peripheral circulation and to identify possible surrogate biomarkers for schizophrenia. In total, 64 analytes were robustly measured with less than 30% missing values. The list of all measured analytes can be found in Supplementary Table S1. Following data quality assessment, normalization and scaling, the analysis of the multiplex immunoassays resulted in the identification of five significantly altered analytes in the PCP rats (p<0.05) (Table 1). These were predominantly interleukins (IL-5, IL-2, IL-1β) as well as fibroblast growth factor-2 (FGF-2) and macrophage inflammatory protein 1α (MIP-1α).

<table>
<thead>
<tr>
<th>Analyte name</th>
<th>UniProt ID</th>
<th>Gene name</th>
<th>Ratio cPCP/CT</th>
<th>p</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-5 (Interleukin-5)</td>
<td>Q08125</td>
<td>Il5</td>
<td>-1.73</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FGF-2 (Fibroblast Growth Factor-basic)</td>
<td>P13109</td>
<td>FGF-2</td>
<td>-1.35</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-2 (Interleukin-2)</td>
<td>P17108</td>
<td>Il2</td>
<td>-1.60</td>
<td>0.0018</td>
<td>0.0397</td>
</tr>
<tr>
<td>MIP-1alpha (Macrophage Inflammatory Protein-1α)</td>
<td>P50229</td>
<td>Ccl3</td>
<td>-1.35</td>
<td>0.0029</td>
<td>0.0472</td>
</tr>
<tr>
<td>IL-1beta (Interleukin-1beta)</td>
<td>Q63264</td>
<td>Il1b</td>
<td>1.10</td>
<td>0.0104</td>
<td>0.1332</td>
</tr>
</tbody>
</table>

**TABLE 1.** Analysis of protein levels in serum of PCP-treated (n=8) and saline-treated rats (n=8) using multiplexed immunoassay. *P*-Values were determined using SAM. FC = Fold change. CT = vehicle-treated rats

Label-free LC-MS$^f$ proteomic profiling of frontal cortex and hippocampus tissue

Total lysis fractions were prepared from frontal cortex and hippocampus tissue of PCP and vehicle-treated rats and analysed by label-free LC-MS$^f$. This resulted in identification of 555 proteins in the frontal cortex and 937 proteins in the hippocampus. Of these, 79 proteins (14 %) were significantly changed due to PCP treatment in the frontal cortex and 501 proteins (53 %) were altered in the hippocampus (Figure 2, Supplementary Tables S3a and S3b). We detected protein level alterations of 22 enzymes in the frontal cortex of which 10 (45 %) catalyze a metabolic reaction, and of 139 enzymes in the hippocampus of which 94 (68 %) catalyze a metabolic reaction. The most prominent proteomic alterations (FC > ± 1.2, p*<0.05) in both regions are shown in Table 1.

**FIGURE 2:** Overview of the proteomic and metabolomic profiling results. Volcano plots of group comparisons showing the adjusted significance values versus fold change distributions. Horizontal grey lines indicate the adjusted p value threshold of 0.05, vertical grey dotted lines indicate a fold-change threshold of 10 %. (Pos.) = positive ion mode. Green dots represent down regulated proteins/metabolites, red dots represent up regulated protein/metabolites. Grey/black dots represent proteins/metabolites not meeting the threshold. (Neg.) = negative ion mode. Full information can be found in the supplementary information.
Proteomics

Frontal Cortex
Identified proteins: 555
Significant proteins: (no fold change cut-off, p<0.05): 37

Hippocampus
Identified proteins: 937

Metabonomics

Frontal Cortex
Identified metabolites:
(pos.) 1057 / (neg.) 60
Significant metabolites: (no fold change cut-off, p<0.05)

Hippocampus
Identified metabolites:
0/31

Shared proteins: 42

Total:
Frontal Cortex: 555 + 37 = 592
Hippocampus: 937 + 0 = 937

Combined:
592 + 937 = 1529
<table>
<thead>
<tr>
<th>Uniprot Entry</th>
<th>Gene name</th>
<th>Protein names</th>
<th>PC</th>
<th>Ratio</th>
<th>Biological functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEUG</td>
<td>Q24940</td>
<td>Neurogranin (Ng)</td>
<td>5</td>
<td>0.80</td>
<td>2.9E-06 Regulates calmodulin affinity for Ca²⁺. Involved in synaptic plasticity and spatial learning</td>
</tr>
<tr>
<td>KIF15</td>
<td>Q7S9P2</td>
<td>Kinesin-like protein KIF15</td>
<td>2</td>
<td>0.82</td>
<td>1.3E-05 Plus-end directed kinesin-like motor enzyme involved in mitotic spindle assembly</td>
</tr>
<tr>
<td>SNAP</td>
<td>P85996</td>
<td>Nap</td>
<td>2</td>
<td>0.83</td>
<td>2.2E-03 Required for vesicular transport between endoplasmic reticulum and Golgi apparatus</td>
</tr>
<tr>
<td>PGB</td>
<td>P55088</td>
<td>Bcan</td>
<td>2</td>
<td>1.28</td>
<td>2.6E-03 May play a role in the adult nervous system during postnatal development</td>
</tr>
<tr>
<td>CLC11</td>
<td>Q08200</td>
<td>C-type lectin domain family 11 member A</td>
<td>2</td>
<td>1.31</td>
<td>2.0E-06 Acts synergistically with other cytokines, including IL-3, GCSF, GMCSF and FLT3 ligand.</td>
</tr>
<tr>
<td>M3K9</td>
<td>Q2U1V8</td>
<td>Map3k9</td>
<td>2</td>
<td>1.20</td>
<td>3.4E-02 Serine/threonine kinase in MAP kinase signal transduction pathway</td>
</tr>
</tbody>
</table>

**TABLE 2:** Most robust differentially expressed proteins identified in the frontal cortex and hippocampus of chronic PCP-treated rats compared to vehicle-treated rats using label-free LC-MS² (>20% change, p*<0.05). PC = peptide count, P = P-value

**Frontal Cortex**

**Hippocampus**
Global metabolic profiling of frontal cortex and hippocampus tissue via UPLC-MS

We employed UPLC-MS for global metabolic profiling analysis of brain tissue. We identified 1057 metabolite features/peaks after filtering based on relative standard deviation across both models and regions. We were not able to identify any significant metabonomic changes in the frontal cortex of the PCP rat model ($p^*<0.05$) but we found 426 significant changed features ($p^*<0.05$) in the hippocampus. The metabonomic profiling findings were consistent with the proteomic profiling results, showing a greater effect of PCP in the hippocampus (Figure 2). For metabolite peak identification, the top 10 significant hits were selected for the hippocampus and identified using HMDB and Pubchem databases (Table 3, Supplementary Table S4a and S4b). Most of the altered identified metabolites were phospho- and glycerolipids. Notably, N-acetyl dopamine was found to be increased in the frontal cortex.

**TABLE 3:** Differentially altered metabolites identified by metabolic profiling of frontal cortex and hippocampal brain tissue from the cPCP rat model. cPCP rats = 10, vehicle-treated rats = 10.
Label-free LC-MS proteome profiling based pathway analysis

Pathway analysis was performed using the total of all changed proteins in the frontal cortex and the hippocampus ($p^{*}<0.05$) regardless of the magnitude of change. Recent studies have shown that even slight variations in the expression of multiple proteins can result in pathway alterations that might underlie complex disorders. Single protein effects can be important when the individual protein effect is strong and there is a small variance across individuals, which is rarely the case in robust homeostatic or physiological systems or disease states. Pathways analysis in combination with quantitative mass spectrometry can help to identify functional links or causality of complex physiological crossstalk in an in-vivo context. The method provides unbiased insights pinpointing pathways underlying physiological changes. Metabonomic profiling results were not included in the
pathway analysis, as data bank searching alone does not allow adequate identification. Therefore, the analytes need to be identified individually based on their fragments.

Using Ingenuity Pathway Analysis we identified a decrease in neurodevelopment associated biological functions in the frontal cortex. The hippocampus was associated with a decreased activation of the biological processes “plasticity of synapse”, “exocytosis of vesicles”, “behaviour” and “spatial memory”, and an increased activation of “movement of rodents”, “paralysis” and “conditioning” (Figure 3A). This matches the reported behavioural readouts of chronic PCP animal models in the literature.$^{65,66}$

GO-enrichment analysis of the proteomic changes revealed that numerous molecular pathways are affected through the chronic PCP treatment (Figure 2C), indicating that changes in one pathway ultimately lead to changes in the whole system. The most robust enriched biological functions across both brain regions were associated with small GTPases and Rho signalling proteins.

We further investigated which pathways appear to be affected by examining the interactome of the altered proteins. This overcomes the limitations to be restricted to the range of detectable proteins in the QTOF study, where certain protein classes (e.g. low abundant proteins, membrane proteins etc.) are frequently not identified and consequently associated pathways will not appear to be enriched. Therefore, we created cytoscape networks based on significantly changed proteins and their interactors using experimentally defined interaction databases for each comparison. GO-term cluster analysis using the reactome pathway information was performed to derive frontal cortex and hippocampus protein networks and an overlap of significant functions was created to identify the most robust pathway signatures across both regions (Figure 2B). The protein changes appeared to reflect changes in postsynaptic NMDAR activation events, including “Ras activation upon Ca$^{2+}$-influx through NMDAR” and “CREB phosphorylation through the activation of Ras or CaMKII” as well as two other pathways downstream of the NMDAR. Furthermore the biological functions of “activation of BAD and BH-3 only proteins” seem to be affected in the frontal cortex. In the case of the hippocampus, a diverse set of other clusters of associated biological functions were found to be enriched in the interactome. This involved AMPA receptor (AMPAR) signalling, kainate receptor signalling, ERK signalling and the TCA cycle.

**FIGURE 2:** Computational Pathway analysis of the chronic PCP-induced proteomic alterations in frontal cortex and hippocampus brain tissue. A) Ingenuity Pathway Analysis (IPA) showing significantly decreased (black bars, minus sign) and increased (white bars, plus sign) biological functions in chronic PCP rat brain regions. Functions ($p$<0.05) are shown with an activation score (z-score) >1 (increased activation) or <-1
(decreased activation). B) Cytoscape generated interaction clusters based on the altered proteins ($p<0.05$) in the frontal cortex and hippocampus. Interaction networks were created using the IMEx databases and ClueGO was applied to identify clusters within. Overlapping GO-terms amongst the networks are indicated by grey shading. C) Functional enrichment analysis of significantly changed proteins in frontal cortex and hippocampus of the chronic PCP rat. Proteins were split into fold-change bins for separate analyses. Colour coded z-score transformed $p$-values indicate the significance of the enrichment for each bin as indicated.
Selected reaction monitoring validation

We used selective reaction monitoring assays to validate the findings of the proteomic profiling analysis and in order to follow up implications of the bioinformatic pathway changes. Therefore we included key proteins of glutamatergic signalling and proteins already known to be affected by PCP treatment\textsuperscript{39, 43} or in schizophrenia. This led to validation of opposite changes in superoxide dismutase and alpha-actinin 1 (ACTN1) in frontal cortex and hippocampus. Furthermore we validated changes in protein DJ1 (PARK7), the astrocytic phosphoprotein 15 (PEA15) and found alterations, although with opposing directional change, in disks large homolog 4 (DLG4), NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUS1) and neurochondrin (NCDN).

**TABLE 4**: Significantly changed proteins identified via label-based LC-SRM in the frontal cortex and hippocampus of chronic PCP-treated rats compared to vehicle-treated rats. Grey bars show consistency between SRM and LC-MS\textsuperscript{E} analysis.
<table>
<thead>
<tr>
<th>Biological Pathway/Function</th>
<th>PCP</th>
<th>Uniprot-ID</th>
<th>Gene name</th>
<th>TPP</th>
<th>Ratio PCP/Ctrl</th>
<th>p</th>
<th>p*</th>
<th>LC-MS†</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-actinin-1</td>
<td>X</td>
<td>Q9Z1P2</td>
<td>Actn1</td>
<td>4/4</td>
<td>0.59</td>
<td>▼</td>
<td>3.51E-06</td>
<td>2.63E-05</td>
<td>(*)</td>
<td>▼</td>
<td>2/4</td>
<td>1.48</td>
</tr>
<tr>
<td>Aspartate aminotransferase, mito (EC 2.6.1.1)</td>
<td>X</td>
<td>P00507</td>
<td>Got2</td>
<td>6/7</td>
<td>1.24 ▲</td>
<td>▲</td>
<td>2.15E-10</td>
<td>3.23E-09</td>
<td>n.s.</td>
<td></td>
<td>5/7</td>
<td>1.01</td>
</tr>
<tr>
<td>Astrocytic phosphoprotein PEA-15</td>
<td>X</td>
<td>Q5U318</td>
<td>Psea15</td>
<td>8</td>
<td>0.61 ▼</td>
<td>▼</td>
<td>3.05E-04</td>
<td>1.20E-03</td>
<td>n.d.</td>
<td></td>
<td>8</td>
<td>1.18</td>
</tr>
<tr>
<td>Catechol O-methyltransferase (EC 2.1.1.6)</td>
<td>X</td>
<td>P22734</td>
<td>Comt</td>
<td>4</td>
<td>0.71 ▼</td>
<td>▼</td>
<td>1.01E-02</td>
<td>2.53E-02</td>
<td>n.d.</td>
<td></td>
<td>3</td>
<td>0.58</td>
</tr>
<tr>
<td>Cofflin-1</td>
<td>X</td>
<td>P45592</td>
<td>Cfl1</td>
<td>7/6</td>
<td>0.85 ▼</td>
<td>▼</td>
<td>1.51E-05</td>
<td>9.07E-05</td>
<td>n.s.</td>
<td></td>
<td>5/3</td>
<td>1.05</td>
</tr>
<tr>
<td>Cornin-1A</td>
<td>X</td>
<td>Q9Z1N1</td>
<td>Cor1a</td>
<td>5/4</td>
<td>1.51 ▲</td>
<td>▲</td>
<td>1.61E-11</td>
<td>4.82E-10</td>
<td>n.d.</td>
<td></td>
<td>5/2</td>
<td>1.03</td>
</tr>
<tr>
<td>Disks large homolog 4</td>
<td>X</td>
<td>P31016</td>
<td>Dlg4</td>
<td>4/3</td>
<td>0.94</td>
<td>▼</td>
<td>2.92E-01</td>
<td>4.87E-01</td>
<td>n.d.</td>
<td></td>
<td>3/3</td>
<td>0.71</td>
</tr>
<tr>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
<td>X</td>
<td>Q66HF1</td>
<td>Ndufs1</td>
<td>6/3</td>
<td>0.59 ▼</td>
<td>▼</td>
<td>2.35E-06</td>
<td>2.53E-05</td>
<td>n.s.</td>
<td></td>
<td>4/5</td>
<td>1.15</td>
</tr>
<tr>
<td>Neurochondrin</td>
<td>X</td>
<td>O35095</td>
<td>Ncdn</td>
<td>4/5</td>
<td>0.95</td>
<td>▼</td>
<td>3.74E-01</td>
<td>5.49E-01</td>
<td>n.d.</td>
<td></td>
<td>4/4</td>
<td>0.94</td>
</tr>
<tr>
<td>Profilin-1</td>
<td>X</td>
<td>P62963</td>
<td>Pfn1</td>
<td>5/4</td>
<td>0.98</td>
<td>▼</td>
<td>6.62E-01</td>
<td>8.28E-01</td>
<td>n.s.</td>
<td></td>
<td>4/4</td>
<td>0.99</td>
</tr>
<tr>
<td>Prohibitin</td>
<td>X</td>
<td>P67779</td>
<td>Phb</td>
<td>4/6/5</td>
<td>1.00</td>
<td>▼</td>
<td>9.70E-01</td>
<td>9.73E-01</td>
<td>n.s.</td>
<td></td>
<td>4/5/5</td>
<td>0.99</td>
</tr>
<tr>
<td>Protein Di-1 (EC 3.4.-.-)</td>
<td>X</td>
<td>O88767</td>
<td>Park7</td>
<td>3</td>
<td>0.47 ▼</td>
<td>▼</td>
<td>1.82E-04</td>
<td>9.12E-04</td>
<td>*▼</td>
<td></td>
<td>4</td>
<td>1.04</td>
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<tr>
<td>Protein Scl25a13</td>
<td>X</td>
<td>F1LX07</td>
<td>Scl25a12</td>
<td>3/4</td>
<td>1.00</td>
<td>▼</td>
<td>9.73E-01</td>
<td>9.73E-01</td>
<td>n.s.</td>
<td></td>
<td>3/3</td>
<td>1.09</td>
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<tr>
<td>Ras-related protein Rab-35</td>
<td>X</td>
<td>Q5U316</td>
<td>Rab35</td>
<td>4/5</td>
<td>0.81 ▼</td>
<td>▼</td>
<td>3.21E-04</td>
<td>1.20E-03</td>
<td>n.s.</td>
<td></td>
<td>3/4</td>
<td>1.12</td>
</tr>
<tr>
<td>Septin-5</td>
<td>X</td>
<td>Q9UM9</td>
<td>Sept5</td>
<td>5/8</td>
<td>0.80 ▼</td>
<td>▼</td>
<td>7.54E-03</td>
<td>2.06E-02</td>
<td>n.d.</td>
<td></td>
<td>4/10</td>
<td>1.46</td>
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<tr>
<td>Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)</td>
<td>X</td>
<td>P07632</td>
<td>Sod1</td>
<td>8/5</td>
<td>0.86 ▼</td>
<td>▼</td>
<td>1.28E-03</td>
<td>3.33E-03</td>
<td>***▼</td>
<td></td>
<td>4/5</td>
<td>1.10</td>
</tr>
<tr>
<td>Vesicular glutamate transporter 1 (VGluT1)</td>
<td>X</td>
<td>Q62634</td>
<td>Scl17α7</td>
<td>8</td>
<td>1.01</td>
<td>▼</td>
<td>8.01E-01</td>
<td>9.00E-01</td>
<td>n.d.</td>
<td></td>
<td>6</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Abbreviations: IPA = Ingenuity pathway analysis, PSEA = Protein set enrichment analysis, UP-ID= uniprot-ID, FC = frontal cortex, TPP = transitions per proteotypic peptide, ▲, unregulated, ▼, downregulated. *, **, and *** ≤0.05, 0.01, and 0.001, respectively. N.s. = not significant, n.d. = not detected. p-Values were determined using SRMstats and corrected for multiple hypothesis testing. (*) to control for multiple hypothesis testing.
Discussion

Currently there is only limited understanding of the molecular pathology underlying schizophrenia. The study of animal models used in drug discovery efforts of schizophrenia can help to decipher the molecular neurobiology of this complex disorder and identify novel targets for improved treatment. However, limited progress has been made in developing novel pharmacotherapies, partly due to the scarcity of well-characterized animal models. In this study, we investigated the response to chronic PCP treatment in rats using a combination of quantitative Omics-based technologies. This combined approach will help to increase confidence in the validity of the model at the molecular level and thus aid drug discovery studies.

This is the largest study of the PCP rat model carried out to date using a combination of omics technologies to analyze distinct brain regions which have been implicated in schizophrenia along with molecular profiling of blood serum to increase our understanding of the associated systemic effects. The serum profiling led to identification of changes in fibroblast growth factor-2 (FGF-2) and four cytokines/chemokines (IL-2, IL-5, IL-1b, MIP-1a). These surrogate markers can be translated to the clinic, where the use of blood serum or plasma can be used for translational studies. After PCP treatment, a general trend towards an anti-inflammatory state was observed with decreased cell-mediated immune responses observed via decreased levels of MIP-1α [chemoattractant of inflammatory cells], IL-6 [T cell maturation] and IL-5 [immunoglobulin secretion and eosinophil activation]. However, an early immune response was also observed through increased levels of IL-1β.

Cytokine abnormalities have been frequently associated with schizophrenia\(^6^7\) and antipsychotic treatment.\(^6^8\) It has been reported that interleukin IL-2 levels are associated with the negative symptoms and cognitive performance.\(^6^9\) IL-1β has been associated with first episode psychosis,\(^6^7\) paranoid schizophrenia\(^7^0\) and a meta-analysis of 23 studies, comprising of 762 subjects showed that antipsychotic treatment significantly reduced plasma level of IL-1β in schizophrenia-spectrum disorders.\(^6^8\) MIP-1α has been associated with different psychiatric disorders, implying a general involvement of chemokine systems with psychiatric diseases.\(^7^1\) In line with this, elevated levels of serum IL-5 have been associated with an increased likelihood to develop MDD.\(^7^2\)

This study is the first to describe a robust decrease of FGF-2 in PCP-treated rats. FGF-2 has been implicated in the pathophysiology of schizophrenia and the mechanism of action of antipsychotic treatment response.\(^7^3\) Furthermore, it has also been implicated in depression\(^7^4\) and as a marker of antidepressant effects.\(^7^5\) Based on the psychotomimetic and antidepressant effect of NMDAR-antagonists, especially ketamine, perturbations of FGF-2 regulation might be relevant for both disorders. Future work needs to clarify if these peripheral changes contribute to the specific
effect of PCP. With respect to the brain pathology, it is likely that FGF-2 levels in serum resemble levels in the brain since this growth factor can cross the blood-brain barrier.\textsuperscript{76, 77} FGF-2 has been implicated in neurogenesis and gliogenesis during development as well as in adulthood\textsuperscript{78-81} via its role as a neurotrophic factor. This is consistent with our findings of a strong PCP effect on the hippocampal proteome and metabonome, with respect to the number of molecular changes. A growing number of studies have shown that pre- and postnatal exposure to inflammatory stimuli can modulate the number of proliferating and differentiating neural progenitors in the hippocampus and this may have an effect on behaviours relevant to psychiatric disorders.\textsuperscript{82}

In general, the brain tissue profiling identified changes in a large range of proteins induced by cPCP-treatment. Many of these proteins were associated with the post-synaptic density and downstream signal transduction pathways of the NMDAR, the primary target of PCP. Bioinformatic pathway analysis of these proteomic alterations revealed an association with events downstream of NMDA-receptor activation, CREB phosphorylation and Ca\textsuperscript{2+}-influx in both brain regions, which demonstrates the validity of the experimental procedures and provides proof-of-concept of the analytical and bioinformatics approach. To confirm the effects on the postsynaptic density, we validated the alterations in the postsynaptic density protein 95 (DLG4) and actinin 1 (ACTN1) via SRM. ACTN1 interacts with the postsynaptic density network and may play a role in NMDA R and AMPAR localization and modulation of these receptors via effects on Ca\textsuperscript{2+}-flux.\textsuperscript{83, 84}

A noteworthy finding of the present study was the greater number of PCP induced proteomic and metabonomic effects in the hippocampus compared to the changes seen in the frontal cortex. This could open up new avenues of research considering that most previous studies on the PCP mechanism of action and schizophrenia pathology has focused on elucidating potential abnormalities in the frontal cortex.\textsuperscript{85} Indeed, hippocampal deficits are an established feature of schizophrenia\textsuperscript{86} as shown by a range of approaches, such as in-vivo (neuropsychology, structural and functional imaging), post-mortem (histology, gene expression and neurochemistry) and animal model\textsuperscript{87} studies. In this investigation, we identified proteomic correlates for behavioural functions based on hippocampus proteomic abnormalities, such as decreased activation of “behaviour” and “spatial memory” derived from the PCP-induced hippocampus proteome alterations as well as increased activation of the biological functions “movement”, “paralysis” and “conditioning”. In the case of the frontal cortex the study was unable to identify any associations of the proteomic alterations with behavioural readouts. The hippocampal alterations were further associated with decreased plasticity of synapse and neurotransmitter release at the proteome level and lipid metabolism at the metabonome level. The hippocampal pathology in schizophrenia appears to be linked with at least some of the cognitive deficits, given the central mnemonic roles of this brain
These key changes most likely reflect alterations in the precise organisation and functioning of neural circuits within this tissue and which connect it with other structures, notably the prefrontal cortex.

*Post-mortem* studies have provided increasing evidence for glutamatergic neurotransmission abnormalities in schizophrenia. Such studies have found hippocampal changes including reduced expression of one or more subunits for all three ionotropic receptors (NMDAR, AMPAR, and kainite receptor). In addition, recently described susceptibility genes for schizophrenia all act upon glutamatergic synaptic transmission, which may therefore be part of the core pathophysiology. We identified PCP-induced alterations in proteins involved in AMPAR trafficking in the hippocampus, which is one of the key mechanisms of synaptic plasticity. Studies have shown that NMDAR channel opening and the subsequent rise in postsynaptic calcium concentration during repetitive synaptic activity, leads to regulated trafficking of postsynaptic AMPARs into and out of excitatory synapses. Targeting AMPAR signalling might therefore, represent a novel target in schizophrenia research.

This study also detected changes in Ca$^{2+}$, opioid, kainate and ERK signalling, which have been previously associated with NMDAR hypofunction. For instance, repeated administration of PCP reduces sigma-1 receptors in the hippocampus. Interestingly, effects on neurotransmitter metabolism were identified in the hippocampus, as shown by IPA and cytoscape pathway analysis. The most robust protein findings were associated with GABA-receptor pathways. A compromised GABAergic system has been hypothesized to be involved in schizophrenia. Notably, NMDAR hypofunction has been proposed to promote deficits in GABAergic signalling and PCP administration during neurodevelopment affects the functionality of GABA interneurons in later life. One action of NMDA antagonists is to reduce the excitation of fast-spiking GABA interneurons, resulting in disinhibition of pyramidal cells. Overactive pyramidal cells, notably those in the hippocampus, can drive a hyperdopaminergic state that produces psychosis.

Finally, we identified alterations in the protein level of the superoxide dismutase enzyme in both the frontal cortex and hippocampus, which is part of the reactive oxygen species (ROS) defense system. Oxidative stress damages many cell structures such as protein, lipids and DNA. This is consistent with our metabonomic profiling analysis which identified abnormalities in lipids and fatty acids. Impairments in energy metabolism are common traits of psychiatric disorders and were previously identified by functional assays, gene and protein expression studies as well as linkage analysis in schizophrenia patients. PCP administration to rats results in reduced rates of oxygen uptake in mitochondria isolated from their brains and a meta-analysis of 44 studies identified a total antioxidant status in serum and plasma as a state marker for first-episode psychosis. For these
reasons, molecules that possess antioxidant and anti-inflammatory properties may be useful as potential novel treatments the first stages of schizophrenia (for review see 102).

This comprehensive proteomic and metabonomic study of the cPCP rat model provides novel molecular evidence showing that different neurotransmitter systems are affected through PCP treatment and these effects occur primarily on the hippocampus. Importantly we were able to find potential molecular correlates which may be linked to the behavioural readouts in this model. Further studies are warranted to investigate this possibility as this could lead to identification of novel therapeutic targets involved in regulation of psychiatric symptoms. Furthermore, understanding the changes in glutamate neurotransmission in schizophrenia may facilitate the discovery of novel targets for pharmacological intervention, which are especially needed for the cognitive and negative symptoms in schizophrenia.

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The authors declare no conflict of interest.

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