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Proteomic investigation of the hippocampus in prenatally stressed mice implicates changes in membrane trafficking, cytoskeletal, and metabolic function.

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Proteomic investigation of the hippocampus in prenatally stressed mice implicates changes in membrane trafficking, cytoskeletal and metabolic function.

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Abstract

Prenatal stress influences the development of the fetal brain and so contributes to the risk of the development of psychiatric disorders in later life. The hippocampus is particularly sensitive to prenatal stress, and robust abnormalities have been described in the hippocampus in schizophrenia and depression. The aim of this study was to determine whether prenatal stress is associated with distinct patterns of differential protein expression in the hippocampus using a validated mouse model.

We therefore performed a comparative proteomic study assessing female hippocampal samples from eight prenatally stressed and eight control mice. Differential protein expression was assessed using 2-Dimensional-Difference-in-Gel-Electrophoresis and subsequent mass spectrometry. The observed changes of a selected group of differentially expressed proteins were confirmed by western blotting.

In comparison to controls, 47 protein spots (38 individual proteins) were found to be differentially expressed in the hippocampus of prenatally stressed mice. Functional grouping of these proteins revealed that prenatal stress influenced the expression of proteins involved in brain development, cytoskeletal composition, stress response and energy metabolism. Western blotting was utilized to validate the changes in Calretinin, Hippocalcin, Profilin1 and Signal transducing adaptor molecule STAM1. Septin5 could not be validated with western blotting due to methodological issues. Closer investigation of the validated proteins also pointed to an interesting role for membrane trafficking deficits mediated by prenatal stress.

Our findings demonstrate that prenatal stress leads to altered hippocampal protein expression, implicating numerous molecular pathways that may provide new targets for psychotropic drug development.

Keywords: proteomics, 2D-DIGE, hippocampus, prenatal maternal stress, trafficking

1 Introduction

Even though the onset of schizophrenia and affective disorders takes place in early adulthood, the underlying cause may have occurred much earlier, possibly already *in utero*. Developmental lesions may be the result of intrinsic (epi)genetic changes, extrinsic stressors, or, most likely, both [1]. Stress exposure of the pregnant mother has been shown to induce alterations in the fetal environment [2-3], and may predispose the fetus to adult psychopathology.

In humans, the type of prenatal stressors is widely variable, ranging from malnutrition to infection and emotional trauma [4]. Likewise, long-term behavioral and psychiatric outcomes of prenatal stress (PS) vary greatly and include attention, motor and cognitive deficits as well as psychiatric disorders [2,5]. Emotional maternal stress, in particular when experienced during early- to mid- gestation, has been shown to increase the risk for the development of depression [6-7] and schizophrenia [8-10] in the adult offspring. However, studies differ in terms of which gender is more vulnerable and with regard to the sensitive time window; some studies even failed to show any effects [11].

Prenatal restraint stress is a well-established animal model that is widely validated in terms of behavior [5,12-15]. In the restraint stress procedure, pregnant dams are restrained in their last week of pregnancy for 45 min in three daily sessions [16]. The model is particularly congruent with the human situation in terms of the increased anxiety-related [17-18], and depressive-like behavior [19-20] observed in the PS offspring, and also shows some overlap with deficits that are typically seen in schizophrenia [21]. Furthermore, similar to human pregnancy, stress hormones from the pregnant mouse can reach the fetal blood circulation, thereby affecting brain development and increasing stress sensitivity [22]. The effects of PS seem to depend on the genetic background of the animal and are predominantly sex-specific [23-25]. In a previous

behavioral study by our group, only the female PS mouse offspring showed a depressive-like phenotype [23].

The aim of this study was to define distinct patterns of differential protein expression in the hippocampus of mice exposed to PS. We selected the hippocampus because it is of major importance in the pathophysiology of both depression [26] and schizophrenia [27]. Furthermore, this brain structure has been shown to be affected by early life stress in humans [28-29]. As the female mice displayed the most pronounced behavioral phenotype in our previous study [23] as well as in a recent microarray study[30], their hippocampal tissue was the focus of the present study. Based on previous proteomic studies in postmortem brains in psychiatric disorders [31-33], we expected that metabolic and cytoskeletal pathways and processes would be dysregulated in the PS mouse model. In order to direct future research, we applied a hypothesis-generating approach in this study, allowing for an extensive discussion on possibly interesting pathways that are affected by PS.

2 Methods

2.1 Animal samples and prenatal stress

Tissue samples used for proteomic analyses were obtained from eight female PS mice that underwent behavioral testing for depressive-like symptoms in a previous study (for details, see Supplementary Methods and [23]). Ethical approval for this study was granted by the Animal Ethics Board of the University of Maastricht.

2.2 Separation of proteins by two-dimensional gel electrophoresis and Image analysis

Samples were processed and separated by 2D-Difference-in-Gel-Electrophoresis (2D-DIGE) as described previously by our group [34-37] (for details see Supplementary Methods). After electrophoresis, scanning of the gels with CyDye-labeled proteins was performed on a Typhoon^{TM}9410 Image scanner (GE Healthcare, UK). Pre-scans were performed to adjust the photomultiplier tube to obtain images with a maximum intensity of 60,000 to 80,000 units. Images were cropped using ImageQuant^{TM} software (GE Healthcare UK), and protein quantification across experimental groups was carried out by Progenesis SameSpots® (Nonlinear Dynamics, UK).

2.3 Statistical analysis

For the 2D-DIGE data, the Progenesis SameSpots software (Nonlinear Dynamics) was used to match and average individual runs for each group, which generated a list of significantly differentially expressed protein spots (ANOVA, $p \leq 0.05$) between the PS and control group.

2.4 Protein Identification by Mass Spectrometry

Statistically significant protein spots were selected for protein identification by Mass Spectrometry based on protein spot intensity. Protein spots were extracted from preparative gels, destained and digested with trypsin as previously described [38]. Mass Spectrometry was

performed on an Agilent 6520 Accurate Mass Q-TOF with the HPLC-Chip Cube and 1200 series HPLC system attached to provide nanoflow separation (for details see Supplementary Methods).

3 Results

3.1 2D-DIGE Analysis

On the master gel image, a total of 1418 protein spots were detected and matched across all gel images and statistically analyzed using ANOVA. In total, 47 protein spots were found to be differentially expressed in the hippocampal tissue of the PS group compared to the control animals. Identified protein spots are indicated on a representative 2D-DIGE gel in Figure 1.

3.2 Functional clustering of differentially expressed proteins

Proteins shown to be significantly altered by 2D-DIGE and identified by mass spectrometry were grouped according to biological function by searching the Uniprot database (http://www.uniprot.org). A list of these findings, including functional ontology of the proteins, can be found in Table 1. The proteins could be subdivided into 7 functional clusters, see Figure 2. The highest number of altered proteins (n=14) were matched to developmental pathways and the cellular stress response (n=9). Energy metabolism (n=8) and protein modification pathways (n=6) were also affected by PS. Of note, proteins with a role in cellular trafficking (n=4), calcium buffering (n=2) and myelination processes (n=2) were found to be differentially expressed in the PS group.

3.3 Validation of proteomic findings

Extensive validation to confirm the findings obtained using 2D-DIGE was conducted using western blotting. See Figure 3 for the details of this validation work.

In brief, the following proteins were assessed using western blotting. These proteins were selected on the basis of antibody availability, fold changes, strength of statistical differences and degree to which proteins represented implicated functional pathways;

Calretinin (CALB2). Results from 2D-DIGE showed an increased expression in the PS group (+1.21 fold; $p<0.01$). An increased expression in the PS mice compared to controls was confirmed ($p<0.05$).

Hippocalcin (HPCA). Results from 2D-DIGE showed an increased expression in the PS group (+1.25 fold; $p<0.05$). Western blotting on individual samples from PS and controls confirmed an upregulation in the PS group ($p<0.05$).

Profilin1 (PFN1). Results from 2D-DIGE showed an increased expression in the PS group (+1.45 fold; $p<0.05$). An increased expression in the PS mice compared to controls was confirmed ($p<0.05$).

Signal transducing adaptor molecule (STAM1). Results from 2D-DIGE showed a reduced expression in the PS group (-1.31 fold; $p<0.05$). A reduced expression in the PS mice compared to controls was confirmed ($p<0.05$).

Septin5 (SEPT5). 2D-DIGE showed a downregulation of 22.6% ($p<0.01$) in the PS offspring. Immunoblot data could not confirm differential expression in Septin5. Since Septin 5 has been reported to have five isoforms in humans - which could result from differences in post-translational modifications, possibly including phosphorylations [36] -, we performed a 2D-western blot (for details, see Supplementary Methods), where the membrane was stained with the same Septin 5-specific antibody that had been used for the 1D- western blot. This confirmed the presence of five Septin5 isoforms, i.e. 5 spots for SEPT5, within the mouse hippocampus (see Figure 4). Consequentially, we went back to the primary analysis and re-examined the data for the four newly identified spots and indeed found one further spot to be minimally downregulated, whereas the other three were moderately upregulated (see Table 2). It is reasonable to speculate that the effect of downregulation of one of these isoforms (as identified by 2D-DIGE) was masked by different changes in the others in immunoblotting, and to the best of our knowledge, no isoform-specific antibodies are available for Septin 5.

4 Discussion

While prenatal maternal stress has known effects on behavior and cognition, the mechanism by which these effects occur is not fully understood. Our results demonstrate that female mice that have been stressed *in utero* show various protein expression differences in the hippocampus in comparison to controls. In total, 47 protein spots - representing 38 individual proteins - were found to be differentially expressed in whole hippocampal lysates of the PS group. Functional clustering of these expression changes indicated alterations in cytoskeletal proteins, metabolism, membrane trafficking and myelination pathways in the brain of PS mice.

4.1 Comparisons to differentially expressed proteins from the literature

To complement the results gathered in our hypothesis-neutral approach, we compared our results to those of previous proteomic animal studies investigating the effects of PS on the hippocampus. We found 13 out of our 38 proteins had been shown to be differentially expressed in in these other animal studies. Furthermore, we also compared our results to those of postmortem studies in humans and observed that seven proteins found to be dysregulated by PS in this study had previously been shown to be dysregulated in majorn psychiatric disorders (for details see Table 3).

4.1.1 Comparison to other proteomic studies

A recent proteomics study [39], looking at the effects of PS on the rat hippocampus, identified 26 differentially expressed proteins. While there was overlap in only three individual proteins between this rat study and our own study, namely Dihydropyramidase related protein-2, Synapsin2, and α-Isocitrate dehydrogenase, there was significant overlap in terms of pathways and processes, as both studies implicated cytoskeletal proteins, energy metabolism, synaptic transduction, and synaptic vesicles.

Ten of the 47 proteins identified in our study were also found to be differentially expressed in a recent proteomic study in human hippocampal tissue from schizophrenia and bipolar disorder patients [33]; these included changes in six cytoskeletal proteins (ACTG1, DPYSL2, DPYSL3, INA, NEFL, YWHAE), two stress response proteins (HSPA8 and PARK7) and two proteins that are involved in energy metabolism (ATP5B and ENO2); for further details see Table 3. Some of these proteins, namely NEFL, DPYSL2, PRDX2 and PARK7, have also been suggested as potential biomarkers in schizophrenia in two recent reviews of proteomic findings [31,40].

4.1.2 Gene expression in the brain following PS

We also compared our results to a recent micro-array experiment using a gene-environment interaction (GxE), i.e. a deficient serotonin transporter (5-Htt) x PS paradigm, based on the same PS mouse model [25]. Five genes that were affected by PS (E) on mRNA level were also observed as protein changes in the same direction in our study (TUBB2a, YHWAE, PSMA5, HSPA9 & MBP). Three genes showed an opposite PS effect (E) in gene expression compared to the protein expression changes found in this study (PFN1, SYN2 & IVD). Of note, MBP was identified as significantly altered in both the E and the GxE condition, as well as on the protein level in our study. A detailed summary is given in Table 3.

4.2 Particular pathways and processes found to be affected after PS

4.2.1 Membrane Trafficking

Roughly 10% of the proteins affected by PS were proteins involved in membrane trafficking. Septin5, which seems to be subject to isoform-specific changes after PS, as verified by our 2D-Western Blot, is an important element in vesicle trafficking, particularly in Clathrin-mediated endocytosis (CME) [36,41-42]. Hippocalcin (HPCA) was upregulated in the hippocampus following PS, as confirmed by Western Blot. HPCA is a neuronal Ca^{2+} sensor thought to influence cognitive processes via regulation of long-term potentiation and depression. Long-term

depression requires the internalization of AMPA and NMDA receptors, both of which are dependent on CME. HPCA binds directly to the clathrin adaptor complex AP2 [43] and is required for the proper formation of the complex, after which CME can be initiated [44-45]. Interestingly, the female PS mice that were analyzed in this study showed significant memory impairment during behavioural testing [23]. These results were comparable to a study in HPCA-deficient mice that displayed a decline in spatial and associative memory [46]. Dysregulation of HPCA thus could contribute to the cognitive deficits associated with affective disorders by disturbing CME and thereby synaptic plasticity.

Profilin1 was upregulated following PS, which could be confirmed by Western Blot. It belongs to a family of small proteins that act as actin polymerization promotors *in vivo* [47-48] and form complexes with regulators of endocytosis, synaptic vesicle recycling and actin assembly. Profilins co-localize with Dynamin1 and Synapsin in axonal and dendritic processes [49] and have roles shaping synaptic structure [50].

STAM expression was reduced following PS, which we observed both using 2D-DIGE and using Western Blot. STAM1 is suggested to function as an adaptor molecule involved in the downstream signaling of cytokine receptors through interaction with ubiquitinated cargo proteins on the early endosome. STAM proteins thus participate in the sorting of cargo proteins for trafficking to the lysosome [51].

4.2.2 Cytoskeletal protein changes

Our results show expression changes in components of the cytoskeleton, such as NEFL, Tubulin subunits and INA. Furthermore, we observed downregulation of DPYSL2 and 3 and upregulation of Profilin1, which may interfere with remodeling of the cytoskeleton during development, a requirement for axon guidance [52-53]. 14-3-3 protein epsilon, found to be

downregulated by PS, plays an essential role in neuronal migration by interacting with NUDEL and DISC1 [54-55]. Of note, SNPs in 14-3-3 proteins (YWHAE, YWHAG) are both associated with severe neurological diseases, therefore alterations may involve nonspecific mechanisms of neuronal pathology. In keeping with our findings, cytoskeletal changes in the brain in affective disorders and schizophrenia are well recognized in many brain areas [32,34-35,56-57], including the hippocampus, [58-59]. Together, these findings point to an effect of PS on neuronal migration and axon guidance.

4.2.3 Metabolic changes and stress response

Energy metabolism was implicated by roughly 13% of the proteins found to be differentially expressed following PS. Four of these, IDH3A, IVD, ALDH1B1 and ATP5B, are mitochondrial proteins. ENO2, for which two isoforms were altered by PS, is involved in glucose metabolism and such changes following PS are in keeping with impaired glucose metabolism in schizophrenia [60-61]. Furthermore, we found proteins involved in stress response, namely HSPA9, HSPA8 and HSP90B1 as well as the Redox homeostasis proteins PARK7, PDIA3 and PRDX2 to be differentially expressed following PS. These changes are likely to reflect sensitivity to mitochondrial stress following PS, findings which are in keeping with alterations observed in affective disorder and schizophrenia [31,62].

4.2.4 Calcium sensing proteins

Calretinin has a similar function to HPCA and was also found to be significantly upregulated in the PS group of this study. In schizophrenia, the focus on the role of calcium-binding proteins, like Calretinin or Calbindin [63], has shifted towards a dysfunction in cell signaling in the GABAergic interneurons of the hippocampus. This is understood to impair synchronized firing of neurons in the hippocampal-prefrontal cortex circuit by destabilizing GABAergic inhibitory signaling [64]. Our findings emphasize the crucial role of Ca^{2+} binding proteins, such as HPCA

and Calretinin, for maintaining cellular homeostasis and demonstrate how this process is an outcome of PS.

4.2.5 Myelin changes

MBP expression was upregulated following PS at both the protein (current study) and mRNA level [25]. These findings are not consistent with myelin gene expression reductions typically observed in schizophrenia and to a lesser extent in affective disorder [65-67] and may be speculated to represent a compensatory reaction to myelin dysfunction such as observed in major psychiatric disorder [68-69]. Interestingly, MBP was also shown to be differentially methylated in a hippocampal DNA methylation screening of PS mice (in preparation).

4.3 Methodological considerations

A primary limitation of this study is exposure of the mice to extensive behavioral testing prior to sacrificing them. Although behavioral task exposure was identical for all animals, this repeated stress exposure may have influenced our results, possibly masking some of the effects that were induced purely by exposing the mice to PS, thus, permanently imprinting on hippocampal protein expression patterns in a condition-dependent manner. Secondly, the lack in overlap between our study and the study published by Mairesse et al. [39], might be explained by the fact that the PS paradigm in rats shows a distinct sex-specificity, with males being more affected by PS exposure whereas in mice females are most affected [23]. Thirdly, our 2D-DIGE results are rather small in fold change. However, spot volume normalization, within a gel and across gels (using the internal standard), add to the sensitivity of the fluorescent dyes and their wide linear dynamic range. Of the five proteins that we attempted to validate using western blotting, we successfully confirmed four. Lack of confirmation of Septin5 may be due to isoform specific changes to which the antibody used in our Western Blot experiments was insensitive. However, we cannot entirely exclude that the Septin5 finding might be a false positive.

4.4 Concluding remarks

In summary, this investigation is the first proteomic study of the hippocampus in PS mice and provides evidence that the mouse model reproduces hippocampal deficits relevant to the pathology of schizophrenia and affective disorders. Our results implicate that PS affects cytoskeletal and synaptic function, energy metabolism, myelination and membrane trafficking. These changes are consistent with changes observed in schizophrenia and affective disorders [31-32] and so our findings strengthen previous evidence that PS in the mouse is a valid animal model for affective disorders and schizophrenia [25]. Furthermore, our work contributes to the focus on new proteins such as Calretinin, Hippocalcin, Septin5, Profilin1, STAM1 and 14-3-3 (YWHAE) and processes such as calcium buffering that may be part of the pathophysiology of affective disorders. The results of this study enhance our knowledge of the nature and extent of protein changes following PS and provide valuable information about the molecular mechanisms that are involved in the severe illnesses that manifest themselves in human PS offspring.

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Contributors

DRC, MJD and MF designed the proteomic study; DLAvdH and JP designed and carried out the mouse PS treatment; MF undertook the proteomics study and analysis, MF and RO did the validation work, managed the literature searches; and the analyses; MF, RO, HWMS, DLAvdH DRC,wrote the first draft of the manuscript.

Conflict of Interest

All authors declare that they have no financial or personal conflict of interest.

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Table and Figure legends

Table 1. Differentially expressed protein spots (ANOVA $p \leq 0.05$) identified by mass spectrometry together with information on their fold change, the functional ontology and grouping of the proteins. ANOVA, analysis of variance

Table 2. Five isoforms of Septin5, one confirmed by mass spectrometry and four additionally by two-dimensional western blot. The altered expression and significance levels of each of these protein spots in prenatal stress in comparison to controls are listed. Protein spot 20 was revealed by primary analysis (significance $p= 0.007$) and spots 506, 840, 938 and 1228 could be traced back to the original 2D-DIGE data after 2D western blot analysis. None of the latter spots gained significance, the majority of the spots were found to be slightly upregulated and one was minimally downregulated. This probably explains why the validation of Septin5 in our sample failed when using 1D western blotting, as there is no marker for specific isoforms of Septin5.

Table 3. Comparison to differentially expressed proteins in the literature. All proteins found to be significantly altered in this study were compared to significant findings in a human study that evaluated protein expression in the hippocampus of schizophrenia and bipolar patients [31] and to mouse micro-array data of prenatally stressed PS mice (E), some of which were genetically manipulated to make them more vulnerable to PS (G) [24]. We also compared our findings to a recent proteomics study investigating rat hippocampus [37]. Furthermore, a recently published article evaluated proteomic studies in schizophrenia brain tissue, listing some of the proteins found in this study as potential biomarkers for schizophrenia [29]. Eight out of the ten proteins involved in cytoarchitectural changes were mentioned in at least one of these publications. Two proteins involved in membrane trafficking could be retrieved in at least one other study, as could one of the proteins involved in protein modifications, three proteins of the cellular response to

stress, four proteins involved in energy metabolism, one myelination protein and one calcium sensor. ↑= upregulated, ↓= downregulated; SCZ = schizophrenia, BP = bipolar disorder; E = environmental factor (here: PS), GxE = gene-environment interaction (here: 5-Htt +/- x PS).

Figure 1. A representative 2-dimensional difference gel electrophoresis gel, pH 4-7, indicating the protein spots found to be significantly altered in PS mice and subsequently identified with mass spectrometry. Protein identification numbers relate to the proteins listed in Table 1. Mw indicates molecular weight.

Figure 2. Chart displaying the categories into which significantly altered protein spots could be grouped. Clustering was done manually using the Uniprot database. CNS = Central Nervous System.

Figure 3. Validation of differentially expressed proteins. * = $p \leq 0.05$. Protein expression changes were assessed by Western Blot analysis of whole hippocampal lysates from eight animals per group. Results were averaged over three consecutive runs for each protein of interest. Bar graphs are presented as percentage of the control group. Error bars indicate standard error of the mean. Next to each graph is a representative image of an immunoblot stained with a specific antibody. Samples were loaded onto SDS-PAGE gels in a randomized order. Immunoblots were incubated with antibodies specific to calretinin at ~ 31 kDa (A), Profilin1 at ~ 15 kDa (B), STAM 1 at ~ 78 kDa (C) and hippocalcin at ~ 22 kDa (D). All membranes were counterstained with an antibody against Erk2 at ~ 43 kDa to control for loading differences. PS = prenatal stress, CALB2 = calretinin, PFN1 = Profilin1, HPCA = hippocalcin.

Figure 4. 2D-Western Blot of Septin5 in whole hippocampal lysate. Tissue was pooled from all samples, separated in two dimensions (pH and molecular weight) and blotted onto a membrane. The protein spot identified as differentially expressed in the primary analysis (number 9) is

1 listed, in addition to those others identified by 2D blot (numbers 506, 840, 938, 1228). See Table
2 3 for fold differences between PS and control groups for these spots (corresponding numbers are
3 used).
4