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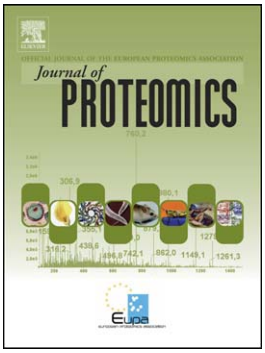

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MATERNAL ALTERATIONS IN THE PROTEOME OF THE MEDIAL PREFRONTAL CORTEX IN RAT

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Abstract

Proteomic differences between rat dams and control mothers deprived of their pups immediately after delivery were investigated in the medial prefrontal cortex (mPFC).

A 2-D DIGE minimal dye technique combined with LC-MS/MS identified 32 different proteins that showed significant changes in expression in the mPFC, of which, 25 were upregulated and 7 were downregulated in dams. The identity of one significantly increased protein, the small heat-shock protein alpha-crystallin B chain (Cryab), was confirmed via Western blot analysis. Alpha-crystallin B chain was distributed in scattered cells in the mPFC, as demonstrated by immunohistochemistry. Furthermore, it was found to be localized in parvalbumin-containing neurons using double labeling. The elevation of its mRNA level in rat dams was also demonstrated via RT-PCR.

The functional classification of the altered proteins was conducted using the UniProt and Gene Ontology protein databases. The identified proteins predominantly participate in synaptic transport and plasticity, neuron development, oxidative stress and apoptosis, and cytoskeleton organization. A common regulator and target analysis of these proteins determined using the Elsevier Pathway Studio Platform suggests that protein level changes associated with pup nursing are driven by growth factors and cytokines, while the MAP kinase pathway was identified as a common target. A high proportion of the proteins that were found to be altered in the mPFC are associated with depression.

Biological significance

The behavior and emotional state of females change robustly when they become mothers. The brain, which governs these changes, may also undergo molecular alterations in mothers. As no proteomics approaches have been applied regarding maternal changes in the brain, we addressed this issue in the mPFC as this brain area is the uppermost cortical center

of maternal control and the associated mood changes. The high number of protein-level alterations found between mothers taking care of their litter and those without pups indicates that pup nursing is associated with cortical protein-level changes. Alterations in proteins participating in synaptic transport, plasticity and neuron development suggest neuroplastic changes in the maternal brain. In turn, the relatively high number of altered proteins in the mPFC associated with depression suggests that the physiological effects of the protein-level alterations in the maternal mPFC could promote the incidence of postpartum depression. Alpha-crystallin B chain, a protein confirmed to be increased during maternal behaviors, was selectively found in parvalbumin cells, which, as fast-spiking interneurons, are associated with depression. The function of alpha-crystallin B chain should be further investigated to establish whether it can be used to identify drug targets for future drug development.

Keywords: *maternal behavior, depression, 2-D DIGE, alpha-crystallin B chain - Cryab, synaptic transport and plasticity, common regulator and target analysis, parvalbumin positive GABAergic interneurons*

Introduction

The behavior and the emotional state of females changes immensely as they become mothers. Female rats avoid or even hurt pups, while mothers take care of their pups and defend them against intruders [1]. Maternal behaviors in postpartum rats include nest building, the retrieval of pups to the nest, the licking of pups, and the adoption of appropriate postures for suckling [2]. These behavioral alterations are accompanied with reduced anxiety- and depression-like behaviors, and increased aggressiveness towards intruders [3-5]. The highest, cortical control influencing maternal responses implies the involvement of the medial prefrontal cortex (mPFC) in maternal adaptations [6-8]. In addition, the mPFC is part of the limbic system and is associated with emotional changes, including anxiety, stress responses, and depression, as well as aggression [9-15]. Impaired maternal responsiveness in human mothers suffering from postpartum depression (PPD) has been shown to be associated with reduced activity in brain areas corresponding to the mPFC [16-18]. Approximately 13% of women experience PPD within 14 weeks after giving birth [19], and 19% of women experience a depressive episode during pregnancy or during the first 3 months postpartum [20]. PPD has both immediate and long-term effects on a mother's mental health, as well as negative effects on the development of their children [21-23]. Women suffering from PPD display a disturbed mother-infant relationship, decreased responsiveness to the demands of parenting, mood disorders, symptoms of anxiety and confusion [24,25]. During the pregnancy and postpartum, mothers have to face new problems. An elevated anxiety and stress level at the time of pregnancy are one of the major risk factors for PPD [26,27]. These stressful factors can increase a mother's vulnerability and the onset of mental illness [28,29].

While the involvement of the mPFC in orchestrating maternal behavior and related emotional changes is well-established, little is known about the underlying molecular mechanisms except that the maternal alterations are not simply due to changes in hormone

levels [2,30,31]. Estrogen and progesterone contribute to maternal adaptations during pregnancy, but they are not required for the maintenance of maternal responsiveness, and even ovariectomized female rats can be induced into a fully maternal state by prolonged pup exposure [1]. Furthermore, motivational and emotional states also change in rats in the postpartum period [32,33]. Therefore, we hypothesized that the behavioral and emotional changes in rat mothers are accompanied by alterations in the brain at the protein level. Because proteomics has not been applied to reveal protein-level alterations in the maternal brain, we compared protein levels in brain tissue homogenates from maternal and non-maternal rats. One group of animals consisted of mother rats 11-12 days after parturition. To focus on protein-level changes associated with maternal care and eliminate pregnancy-induced alterations, the control group consisted of mother rats whose litter was removed immediately after delivery. These rats no longer show maternal behaviors by the time the brain tissue samples were collected [34], but they went through pregnancy, parturition and all of the associated hormonal changes prior to litter removal.

Methods

Animals

Animals were kept under standard laboratory conditions with 12-hour light and dark periods (lights were on from 08.00 am to 08.00 pm). Food and water were supplied *ad libitum*. The care and experimentation of all animals conformed to the Hungarian Act of Animal Care and Experimentation (1998, XXVIII) and to the guidelines of the European Communities Council Directive, 86/609/EEC as well as with local regulations for the care and use of animals for research.

A total of 46 adult Wistar rats were used in the study. The number of pups of rat dams was adjusted immediately after parturition. In the maternal groups, the litters were adjusted to

8 pups, and pups were removed from the control groups (pup-deprived mothers). For perfusions and dissections, rats were anesthetized with an intramuscular injection of anesthetic mix containing 0.2 ml/300 g body weight ketamine (100 mg/ml) and 0.2 ml/300 g body weight xylazine (20 mg/ml) and sacrificed at 11-12 days postpartum. For proteomics, Western blot and RT-PCR analyses, the mPFCs were dissected from the freshly removed brains. For immunohistochemistry, the rats were transcardially perfused with saline followed by 4% paraformaldehyde.

Microdissection of brain tissue samples

The brains of 20 mothers with litters and 20 pup-deprived control rats (mothers whose pups were removed immediately after parturition) were dissected. Coronal brain sections (2-mm thick) were prepared with a razor blade. The anterior level of the optic chiasm (bregma level: +0.3 mm) was used to determine the antero-posterior levels. For the dissection of the mPFC sample, first a coronal section was cut between bregma levels 4.3 and 2.3. Subsequently, the ventral 1.5 mm of this section containing the anterior olfactory nucleus was removed, and then, vertical cuts were made 1 mm lateral to the midline to include the mPFC from both sides of the brain (Figure 1). The dissected tissue samples were quickly frozen on dry ice, and stored at -80 ^{o}C.

Proteomic analysis via two-dimensional differential gel electrophoresis (2-D DIGE)

For the proteomic analysis, mPFC dissected from 6 mothers with litters and 6 pup-deprived control rats were used. The details of the 2-D DIGE protocol has been described in our previous study [35]. The 2-D DIGE minimal dye labeling method was used. Equipment and software were supplied by GE Healthcare, Little Chalfont, UK. Briefly, proteins from the homogenized brain tissues were acetone-precipitated and then re-suspended in a lysis buffer

containing 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris and 5 mM magnesium-acetate. The pH of the samples was adjusted to 8.5, and the protein concentrations were determined with a 2-D Quant Kit. Samples (50 µg) were labeled using a CyDye DIGE Fluor Minimal Labeling Kit according to the manufacturer's instructions. The maternal and pup-deprived tissue samples were randomly labeled with Cy3 and Cy5, and the internal standard (a pool of equal amounts of all samples from the experiment) was labeled with Cy2 fluorescent dye. The three differently labeled protein samples were merged, and six mixtures (six simultaneous gels) were run. Isoelectric focusing was performed on 24 cm IPG strips (pH 3–10 NL) for 24 h in an Ettan IPGphor instrument to attain a total of 80 kVh. The voltages applied in each mode were as follows: 30 V for a 3 h step, 500 V for a 5 h gradient, 1,000 V for a 6 h gradient, 8,000 V for a 3 h gradient, and 8,000 V for a 6 h step. Focused proteins were reduced and then alkylated in equilibrating buffers containing mercaptoethanol and iodoacetamide, respectively, for 20–20 min. Subsequently, the IPG strips were loaded onto 10% polyacrylamide gels (24×20 cm), and SDS-PAGE was performed using an Ettan DALT Six System. Gels were scanned with a Typhoon TRIO+ scanner, selecting appropriate lasers and filters. Gel images were visualized using ImageQuant TL software. A differential protein analysis was performed using DeCyder 2D 7.0 software with the Differential In-gel Analysis (DIA) and Biological Variance Analysis (BVA) modules. The internal standard contained equal amounts from the same sample in all gels, and the changes in fluorescence intensity of the protein spots were normalized to the values of the corresponding internal standard. Independent Student's *t*-tests were performed to determine the statistical significance of changes in protein abundance, with a cut-off of $p < 0.05$ for each protein spot.

Preparative two-dimensional gel electrophoresis for protein identification

For the identification of proteins in the spots of interest, a separate preparative two-dimensional gel electrophoresis was performed using a total of 800 µg of protein per gel. Resolved protein spots were visualized using Colloidal Coomassie Blue G-250 (Merck, Darmstadt, Germany).

Protein identification by mass spectrometry

2-D DIGE gel-separated protein spots were cut from the gel and in-gel digested for mass spectrometry-based protein identification using the protocol available on-line (http://msf.ucsf.edu/protocols.html). After reduction with dithiothreitol (DTT) and alkylation with iodoacetamide (IAM) the proteins were digested with trypsin (sequencing rate modified trypsin from pig pancreas, Promega). Tryptic peptides were subjected to LC-MS/MS analysis on an LCQ-Fleet ion trap mass spectrometer (Thermo) coupled in-line with a nanoAcquity HPLC system (Waters). Peptide extracts (5 µl of the 10 µl sample) were injected into the nano-HPLC system using a trap column (Symmetry C18, 0.18×20 mm, 5 µm, Waters) and were analyzed in a BEH300C18 1.7 um (0.1 × 100 mm) nanoAcquity UPLC column (Waters) using a gradient elution (10-40% B solution over 30 minutes, B solution: 0.1 % formic acid in acetonitrile, A solution: 0.1% formic acid in water). MS data were acquired in a data-dependent fashion using a triple play method, with each survey scan followed by zoom scans and CID scans (normalized collision energy: 35) of the 3 most abundant, multiply charged precursor ions. Dynamic exclusion was set to 30 sec. Mascot Distiller (ver: 2.2.1.0) was used to generate the MS/MS peak list files (mgf) from the raw data and the ProteinProspector (v. 5.3.0.) search engine was used for the database search. We used the following parameters for the search: database: UniProtKB.2011.03.30 HUMAN RODENT (218476/14423061 entries searched); enzyme: trypsin with maximum 2 missed cleavage sites; fixed modifications: carbamidomethyl (C); variable modifications: acetyl (protein N-term), Gln->pyro-Glu (N-

term Q), oxidation (M); peptide mass tolerance: ± 0.6 Da; fragment mass tolerance: ± 1 Da. Proteins identified with at least with 2 unique peptides (minimum peptide score: 15) were considered as a valid hit.

Functional clustering

Significantly altered proteins were clustered on the basis of the protein annotations in the UniProt (http://www.uniprot.org/) and Gene Ontology (http://geneontology.org/) databases. The proteins were clustered into groups according to their most relevant cellular functions.

Bioinformatic analysis of significant protein changes

The interactions between significantly altered cortical proteins were analyzed using the Elsevier Pathway Studio Platform. We selected common regulator and target proteins having a minimum of 4 relationships with the significantly altered maternal proteins from the experimental results. For further analysis, common regulators and targets connected with Cryab were selected.

Validation of the significantly altered proteins via Western blot (WB) analysis

For Western blotting, mPFC dissected from 6 mothers with litters and 6 pup-deprived control rats were used. The protein with highest fold change in expression level in the mPFC was shown to be alpha-crystallin B chain (Cryab), thus, we selected it for validation via WB analysis. We used the same samples that were utilized in the 2-D DIGE method. Both maternal and pup-deprived samples (n=6-6) weighed 100 µg. Proteins were separated with Tricine-SDS-polyacrylamide gel electrophoresis on 15% polyacrylamide gels and then transferred to Hybond-LFP PVDF transfer membranes (GE Healthcare). The membranes were

blocked with 5% BSA in Tris-buffered saline with 0.1 % Tween 20 (TBS-T). The blots were incubated with the 1:1 mixture of goat anti-Cryab primary antibody (sc-22391, Santa Cruz Biotechnology) at a 1:500 dilution and mouse anti-CoxIV primary antibody at a 1:1,000 dilution (sc-58348, Santa Cruz Biotechnology). Subsequently, the membranes were washed for 4 x 5 min in TBS-T followed by an incubation with a 1:1 mixture of CruzFluor 488 conjugated anti-goat (1:1,500 dilution, Santa Cruz Biotechnology) and Cy3 conjugated anti-mouse IgG secondary antibodies (1:2,500 dilution, GE Healthcare). After washing the membranes in TBS-T and then in TBS, the bands were visualized using a Typhoon TRIO+ scanner. Fluorescence intensities were quantified using ImageQuant TL software. The densitometry data for the intensity of each protein band were analyzed with ImageJ software (NIH, Bethesda). Densitometric values of the Cryab protein bands were normalized to the densities of the CoxIV loading control protein bands of the same sample. Differences between maternal and pup-deprived samples were statistically analyzed using independent two-tailed Student's *t*-test (Prism 5 for Windows, GraphPad Software, Inc., La Jolla, CA).

Quantitative RT-PCR (qRT-PCR)

For qRT-PCR analysis, mPFC dissected from 8 mothers with litters and 8 pup-deprived control rats were used. A quantitative real-time RT-PCR analysis was performed for Cryab in the maternal and pup-deprived mPFC samples as described previously [36]. The total RNA was isolated from the microdissected mPFC using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After diluting RNA to 2 µg/µl, it was treated with Amplification Grade DNase I (Invitrogen) and cDNA was synthesized with a Superscript II reverse transcriptase kit (Invitrogen), according to the manufacturer's instructions. After a 10-fold dilution, 2.5 µl of the resulting cDNA was used as a template in PCR reactions using SYBR Green dye (Sigma, St. Louis, MO). The PCR reactions were

performed with a CFX96 Real-time System (Bio-Rad Laboratories, Hercules, CA) using iTaq DNA polymerase (Bio-Rad Laboratories) in total volumes of 12.5 µl under the following conditions: 95 ^{o}C for 3 min, followed by 40 cycles of 95 ^{o}C for 0.5 min, 60 ^{o}C for 0.5 min and 72 ^{o}C for 1 min. There were 4 genes, which did not change between the mother and control groups, used as references: Beta actin (Actb), Cytochrome c oxidase subunit IV isoform 1 (Cox4i1), Glyceraldehyde-3-phosphate-dehydrogenase (Gapdh), and Lactate dehydrogenase A (Ldha). The GenBank accession numbers and the primers used in the study are listed in Table 1. Primers were used at a final concentration of 200 nM. Cycle threshold values (C_T values) were obtained from the linear region of the baseline-adjusted amplification curves using Bio-Rad CFX Manager software (Bio-Rad Laboratories). The dC_T method was used to calculate the changes in the expression of Cryab in relation to the average of the 4 reference genes. Statistical comparisons (Prism 5 for Windows, GraphPad Software, Inc) were made using Student´s t-test.

Immunohistochemistry (IHC)

For the immunohistochemistry, mother and pup-deprived rats (3-3) were deeply anesthetized and fixed with 4% paraformaldehyde via transcardially perfusion. Brains were removed and postfixed in 4% paraformaldehyde for 24 h then transferred to 20% sucrose in phosphate buffer (PB, pH=7.4) for 48 h. Serial coronal brain sections were cut at 40 µm on a sliding microtome. Sections were collected in PB containing 0.05% sodium-azide and stored at 4 ^{o}C. The mPFC sections were incubated in anti-Cryab primary antibody (1:20 dilution) followed by a biotinylated anti-goat secondary antibody (1:1,000 dilution, Jackson Immunoresearch, West Grove, PA). Visualization was performed using an ABC kit (1:500 dilution, Vector Laboratories, Burlingame, CA, USA) and a Ni-DAB reaction. For double labeling, the visualization of Cryab was performed using FITC-tyramide (1:8,000)

amplification followed by incubation with mouse anti-calbindin (1:900 dilution, catalogue number: C9848, Sigma) or anti-parvalbumin (1:800 dilution, catalogue number: P3088, Sigma) antibodies, which were visualized with Alexa 594-conjugated anti-mouse IgG (1:400 dilution, Thermo Fisher Scientific, Waltham, MA). Subsequently, the sections were mounted, dried, and coverslipped in antifade medium (Prolong Antifade Kit; Molecular Probes).

Histological analysis

Sections were examined using an Olympus BX60 light microscope equipped with fluorescent epi-illumination. Images were captured at a resolution of 2048 by 2048 pixels with a SPOT Xplorer digital CCD camera (Diagnostic Instruments, Sterling Heights, MI). Confocal images were acquired with a Nikon Eclipse E800 confocal microscope equipped with a BioRad Radiance 2100 Laser Scanning System using 20-60 X objectives at an optical thickness of 1-3 µm.

The contrast and sharpness of the images were adjusted using the "levels" and "sharpness" commands in Adobe Photoshop CS 8.0. The full resolution of the images was maintained until the final versions were adjusted to a resolution of 300 dpi.

Results

Proteomic identification of maternally altered proteins

We detected approximately 1,200 quantitatively measurable spots per gel with 2-D DIGE (Minimal Dye labeling) in the mPFC samples from mother and pup-deprived rats. There were 45 different protein spots that showed a significant change, among which, 37 showed higher and 8 showed lower fluorescence intensities in dams than they did in the

control animals. Representative 2-D DIGE gel images with differentially expressed protein spots from the mPFC samples are shown in Figure 2. Fold changes in the fluorescence intensities between the spots of samples collected from mother and pup-deprived rats were in the range of -1.60 to 1.86 (Figure 3A). Proteins in the significantly changed spots were identified via HPLC-MS/MS. We were able to describe a total of 32 differentially expressed proteins in the mPFC (Table 2). Several proteins were present in more than one spot, suggesting post-translational modifications or the presence of protein isoforms. Cryab showed the greatest increase in protein level in the mPFC (+1.86) samples from dams. The greatest decrease in protein level was shown by tubulin-specific chaperone A (Tbca, -1.60).

Functional clusters of maternally altered proteins

The identified, altered mPFC proteins participate in a variety of cell processes, including synaptic transport and plasticity (n=6), neuron development (n=5), protein synthesis and folding (n=3), cytoskeleton organization (n=3), response to oxidative stress and apoptosis (n=3), cell differentiation (n=2), amino acid metabolism (n=2), cell cycle (n=2), glucose metabolism (n=2), protein transport (n=1), transcription (n=1), protein degradation (n=1) and ion transport (n=1), according to the Gene Ontology and UniProt classification (Figure 3B).

Common regulators and targets of maternally altered proteins

In the region of the mPFC, the major Cryab-related common regulators were β-nerve growth factor (NGF), insulin-like growth factor I (IGF1), interleukin-1 β (IL-1B), tumor necrosis factor (TNF), transcription factor Sp1 (SP1) and cellular tumor antigen p53 (TP53), while the major common targets were RAC-alpha serine/threonine-protein kinase (AKT1), insulin (INS), mitogen-activated protein kinase 3 (MAPK3) and mitogen-activated protein kinase 1 (MAPK1) (Figure 4).

Validation of increased level of Cryab

A WB analysis was performed for Cryab in the mPFC (Figure 5). The protein levels of Cryab were significantly increased in dams when compared with levels in control females (1.43 ± 0.14, $p < 0.01$). The normalized Cryab values (Cryab / CoxIV ratio) were also significantly increased in dams, showing a smaller extent of increase but at a higher significance level (1.25 ± 0.03, $p < 0.001$). Thus, the results of the WB analysis of the Cryab protein confirmed the 2-D DIGE data.

Gene expression level of Cryab

Mother rats had a 1.63-times higher level of Cryab mRNA than age-matched pup-deprived female rats in their mPFC samples (Figure 6). The mRNA level of Cryab (expressed as the Cryab/average reference gene mRNA ratio) was 0.72 ± 0.10 and 0.44 ± 0.07 in dams and pup-deprived females, respectively. These results represent a significant increase in the Cryab mRNA level in dams (*t*-test, $p < 0.05$).

Localization of Cryab

We also analyzed the distribution of the Cryab protein in the region of the mPFC of maternal and control brains. The density of Cryab-positive cells was higher in the maternal mPFC. These cells were most abundant in layer IV of the prelimbic (PrL) and infralimbic (IL) cortices. Cryab immunoreactivity was present in parvalbumin-positive GABAergic interneurons but not in calbindin-positive cells (Figure 7). The labeling was most intense in the perisomatic region of the cells.

Discussion

We present here the first proteomic study of maternal changes in the region of the mPFC. First, we discuss the altered proteins based on their functional classifications, including the relation of the altered medial prefrontal proteins to depression. Subsequently, we focus on a particular protein, Cryab, and the consequences of its localization in the mPFC. Finally, the common regulators and targets of the altered proteins are analyzed.

Potential functions of the altered proteins

Most of the altered proteins identified in rat dams are involved in synaptic transport and plasticity (19%), neuron development (16%), the oxidative stress response and apoptosis (10%) and cytoskeleton organization (10%) (Figure 3B). These data suggest remarkable synaptic alterations, which could be related to the flexibly plasticity of the cerebral cortex or the high number of synapses present. Furthermore, the neuronal processes similar to those active during development showed an elevated activity level in rat dams.

The physiological changes that occur in the brain during motherhood require elevated protection against stress factors, explaining the oxidative stress- and cytoskeleton organization-related protein changes. Many of the significantly altered proteins in the mPFC have been suggested to be involved in depression as their level or other properties (e.g. state of phosphorylation) have been reported to change in conjunction with human depression or rodent depression-like behavior (Table 3). Of the 11 proteins associated with depression that we identified as altered in the maternal mPFC, 9 show decreased levels in depressed humans or other animals (Table 3). Interestingly, 8 (Cplx1, Cplx2, Nrgn, Ina, Cryab, Ddah1, Glul, Stam) of these 9 proteins were present at increased levels in the mPFC of rat dams. The Cryab chaperone protein has been shown to have changes in its phosphorylation status in dorsolateral prefrontal cortex tissues from patients with major depressive disorder [37]. The

expression of complexin-2 (Cplx2), a synaptic vesicle clustering regulator, is decreased in the anterior cingulate cortex [38], and the enzyme glutamine synthase (Glul) shows decreased expression levels in the cerebral cortex of patients with major depressive disorder [39]. Neurogranin (Nrgn), a synaptic plasticity regulator, shows a decreased protein level in the hippocampus and prefrontal cortex in association with [40], and decreased protein levels of N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 (Ddah1), a nitric oxide regulator, are also associated with depression-like behavior [41]. RCG55706 (Stam), a protein transport regulator, shows decreased protein levels in mice hippocampi after exposure to prenatal stress [42], while alpha-internexin (Ina), a neuronal morphogenesis-regulating intermediate filament, shows decreased protein levels in rat hippocampi following exposure to stress induced by terrifying sounds, and both of these responses are risk factors for depression [43].

Thus, maternally increased proteins typically show reduced levels in depression. These proteins could contribute to the anti-depression-like traits observed in rat dams [32,44]. Furthermore, the potential maladaptation of some of these proteins during the maternal process could promote the development of depression in human mothers, who have a much higher incidence of depression in the postpartum period (~13%) than does the general population [19,20]. The value of the correlations demonstrated in this study is increased by the lack of previously available specific molecular markers of postpartum depression [45-47].

Alpha-crystallin B chain (Cryab) and its potential involvement in maternal adaptations

Cryab showed the highest increase in protein level in the mPFC of rat dams. Therefore, we further analyzed its expression level using independent methods, and not only validated the initial demonstration of its change at the protein level but also showed that it is elevated at the mRNA level. Thus, Cryab could become a specific molecular marker of postpartum depression, which is missing at present [45-47]. Cryab is a member of the small

heat shock protein (HSP20) family, whose members form large heterooligomeric complexes. Cryab also acts as a molecular chaperone. It does not renature and release proteins, but rather holds them in large soluble aggregates [48]. The distribution of Cryab shows a high degree of heterogeneity: it is most abundant in the lens, and smaller amounts have also been described in other tissues, such as those of the heart, skeletal muscle and the brain [49]. The elevated expression of Cryab has been found in some neurological diseases where it has been suggested to have a neuroprotective function [50,51], although little is known about its protective mechanisms [52]. One suggestion relates to the phosphorylation of Cryab, which occurs when the cells are exposed to various types of stresses [53]. The phosphorylated forms play neuroprotective, anti-apoptotic roles and increase neuronal survival after injury [54]. The cytoprotective effects of Cryab are reflected by its capacity to suppress the aggregation of denatured proteins, which in turn provides anti-apoptotic protection [48,52]. An alternative explanation for the neuroprotective function of Cryab is its modulatory action on lipid membrane polymorphism and fluidity [55]. Our current findings provide the first evidence that Cryab is elevated in the brains of rat dams. In fact, we identified Cryab from two different protein spots in the mPFC, which may be a result of different phosphorylation states of the protein (Figure 2). The increased expression of Cryab in the mPFC of rat dams suggests a higher level of protection against stress factors in this brain region. Immunohistochemical results showed that the density of Cryab-positive cells was the highest in layer IV of the prelimbic and infralimbic cortices and that Cryab immunoreactivity showed membrane-associated localization. We also demonstrated that Cryab is present in parvalbumin-positive (PV), and likely GABAergic, fast-spiking interneurons in the mPFC [56]. These cells are essential for normal brain function as they can regulate the activity of principal neurons. PV interneurons synapse on the cell bodies and axon initial segments of several pyramidal cells to regulate their output. Thereby, they can coordinate the activity of large networks in the brain

[57-61]. The increased neuroprotection of PV GABAergic interneurons might contribute to preserving the function of the medial prefrontal network under stressful conditions in mother rats. Fast-spiking PV interneurons are downregulated in a genetic mouse model of depression, causing low-gamma oscillation disturbances and depression-like behavior [62]. The increased Cryab protein level in PV interneurons and the huge number of proteins that show increased levels in dams suggest an elevation in chaperone activity in PV cells. The insufficient activity of Cryab in cells involved in depression can thus become a cause of depressive behavior during the postpartum period. Examining the putative involvement of Cryab in the development of postpartum depression is an intriguing future research goal as Cryab.

Common regulators and targets of the maternally altered proteins

The common regulator and common target analyses were carried out for all altered proteins, albeit with a focus on connections to Cryab. The major common regulators were NGF and IGF1 (growth factors), IL-1B and TNF (cytokines), SP1 (a transcription factor) and TP53 (a tumor suppressor), suggesting that these regulators play significant roles in the adaptation of the maternal mPFC at the protein level (Figure 4A). Specifically, growth factors and cytokines influence maternal protein changes, which has not been reported before. However, it is becoming increasingly accepted that depression is correlated with the dysregulation of immune processes [63]. IL-1B and TNF, both proinflammatory cytokines, are known to show elevated levels in association with depression [64]. In addition, IL-1B may have an indirect link with postpartum depression through its association with fatigue [65]. Thus, IL-1B could take part in the pathophysiology of postpartum depression. Growth factors have also been implicated in depression [66], as depression-like behavior has been associated with reduced NGF and IGF1 levels in the rat brain [67,68]. Altered NGF levels have been shown to increase the risk of developing depression after chronic stress [69], while

antidepressant drugs have been shown to have a beneficial impact of on the malfunction of IGF1 in adult rats [70]. NGF levels of mothers with postpartum affective disorders are also correlated with infant development [71]. The potential effects of NGF and IGF-1 in the brain of mothers could be mediated by the altered levels of the proteins we identified in the present study.

The major common targets identified in the mPFC were AKT1, MAPK1, MAPK3 (protein kinases) and INS (a hormone) (Figure 4B), suggesting the involvement of these proteins, and particularly the mitogen activated protein kinase (MAPK) pathway, which is a major signaling pathway involved in neuronal plasticity, function and survival in maternal adaptations. While the involvement of this pathway in maternal processes has not been demonstrated, it has been reported to be associated with depression [72,73]. The inhibition of ERK signaling, which initiates the MAPK pathway, can lead to anxiety and depressive behaviors [74]. The mRNA level of MAPK1 has been shown to be altered by both nortriptyline and escitalopram, which are both used as antidepressants [75]. MAPK phosphatase-1 (MKP-1), the key negative regulator of the MAPK cascade, shows a significantly increased expression in postmortem hippocampal tissues of patients with major depressive disorder [76]. In addition, MKP-1 mRNA levels have been shown to be increased in rat and mouse depression models, while antidepressant treatment normalizes its expression [76]. Thus, the MAPK pathway could also play a role in postpartum depression.

Conclusion

Pup nursing is associated with altered levels of a number of proteins in the mPFC. Neuronal development and plasticity are the major functional classes to which the altered proteins belong. Several maternally altered proteins have been shown to be regulated by growth factors, cytokines and signal transduction pathways, while their common targets are

some members of the MAP kinase pathway, suggesting their role in the regulation of maternal responsiveness. The greatest change in protein level was observed for alpha-crystallin B chain (Cryab), which was found to be expressed in parvalbumin-positive neurons, suggesting its role in the maternal adaptation of parvalbumin-positive fast-spiking GABAergic interneurons. Several proteins that showed increased levels in rat dams in the region of the mPFC are known to be downregulated in association with depression, suggesting an anti-depression-like effect in the dams. In particular, Cryab is a potential candidate for involvement in mood changes in mothers.

Acknowledgements

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Figure legends

Fig. 1. Dissection of the medial prefrontal cortex (mPFC) samples. A: The position of the coronal section cut as a first step of the mPFC dissection is shown by the 2 vertical lines on the schematic side view of the brain. B: The mPFC tissue sample was obtained from the coronal section by cuts at the solid lines. Further abbreviations: ac – anterior commissure, AON – anterior olfactory nucleus, cc – corpus callosum, Ins – insular cortex, M – motor cortex, Pir – piriform cortex.

Fig. 2. Representative 2-D DIGE gel image with labeled locations of significantly altered medial prefrontal cortical protein spots. The spot number and the most prevalent identified gene in a particular spot are shown. Red and blue circles indicate significantly increased and decreased changes in protein spots of dams relative to those of the control animals, respectively.

Fig. 3. Fold changes (A) and functional clustering (B) of maternally altered proteins in the medial prefrontal cortex. Proteins with fold changes greater than 1.5 are labeled with yellow.

Fig. 4. Common regulator (A) and target (B) analysis of medial prefrontal cortical proteins, with a focus on those connected to Cryab. *Common regulators:* NGF: beta-nerve growth factor, IGF1: insulin-like growth factor I, IL-1B: interleukin-1 beta, TNF: tumor necrosis factor, SP1: transcription factor Sp1, TP53: cellular tumor antigen p53. *Common targets:* AKT1: RAC-alpha serine/threonine-protein kinase, INS: insulin, MAPK3: mitogen-activated protein kinase 3, MAPK1: mitogen-activated protein kinase 1. Edges indicate the relationships between common regulators/targets and altered maternal proteins. Red and blue

indicate maternal proteins that were significantly increased and decreased, respectively, that have common targets or regulators. Full protein names are presented in Table 2. Green indicates common regulator (A) or target (B) proteins.

Fig. 5. Alteration in the Cryab protein and Cryab/CoxIV ratio in the medial prefrontal cortex of mother and pup-deprived control rats. The level of Cryab protein is significantly higher in mothers than in pup-deprived controls. Left: Densitometric values for Cryab and the Cryab/CoxIV ratio (n=6-6, Student's *t*-test, **: $p < 0.01$; ***: $p < 0.001$, means ± s.e.m. are shown). Right: representative immunopositive bands of Cryab and CoxIV.

Fig. 6. Alteration in Cryab mRNA expression in the medial prefrontal cortex of mother and pup-deprived control rats. The level of Cryab mRNA is significantly higher in mothers than in pup-deprived controls. mRNA expression data are expressed as the ratio of Cryab mRNA to the average of Actb, Cox4i1, Gapd and Ldha mRNA levels (n=8-8, Student's *t*-test, *: $p < 0.05$; means ± s.e.m. are shown).

Fig. 7. Distribution of Cryab immunoreactivity in the medial prefrontal cortex (mPFC). Cryab-labeled cells are shown in the maternal (A) and pup-deprived (control) female rats (B). The cells are abundant in layer IV of the prelimbic (PrL) and infralimbic (IL) cortices. Cryab colocalizes with parvalbumin (C) but not with calbindin (D). White arrows point to double-labeled cells, white arrowheads point to single-labeled Cryab-positive cells, and black arrowheads point to single-labeled calbindin-positive calbindin-positive cells. Scale bars: 500 μm (A, B), 100 μm (C, D).

Table 1. The primers used for amplifying genes in the qRT-PCR study. The GenBank accession numbers and the position of the primers in the sequences are also indicated.

Table 2. The functional clusters of significantly altered proteins in the medial prefrontal cortex of mother rats. The color gradient from red (elevated protein level) to blue (reduced protein level) is used to show the differential abundances of the maternally altered proteins (the numbers represent the average ratios). Abbreviations: Acc: accession number, AR: average ratio, UP: unique peptides number, SC%: sequence coverage percentage, MW: molecular weight, pI: isoelectric point.

Table 3. Significant changes in the levels of proteins in the medial prefrontal cortex that are involved in depression-like behavior. The color gradient from red (elevated protein level) to blue (reduced protein level) is used to show the differential abundances of maternally altered proteins (the numbers represent the average ratios). Abbreviations: Acc: accession number, AR: average ratio. (References: 1-[77], 2-[37], 3-[78], 4-[79], 5-[38], 6-[80], 7-[40], 8-[81], 9-[82], 10-[83], 11-[43], 12-[42], 13-[84], 14-[41], 15-[39], 16-[85]).

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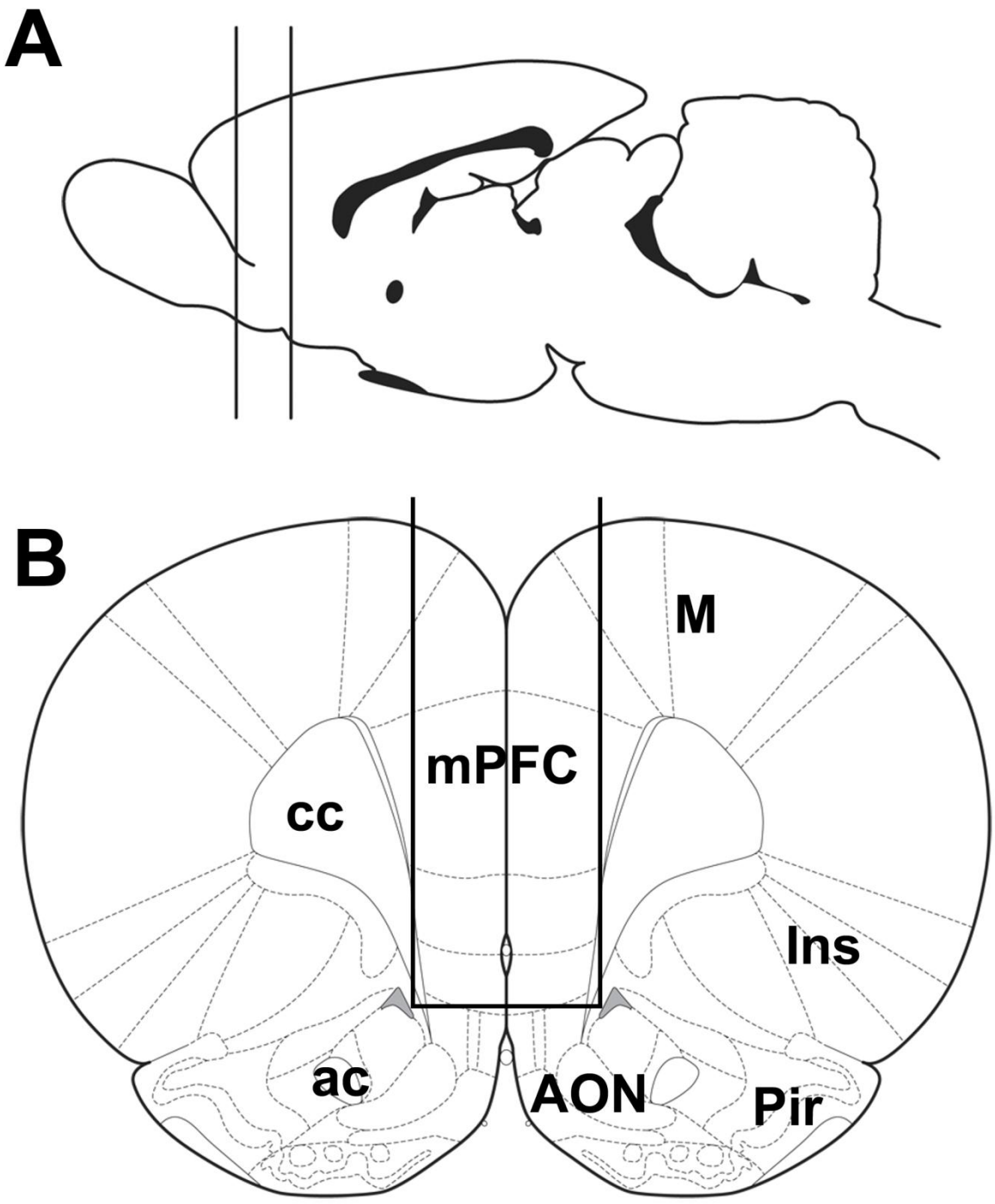
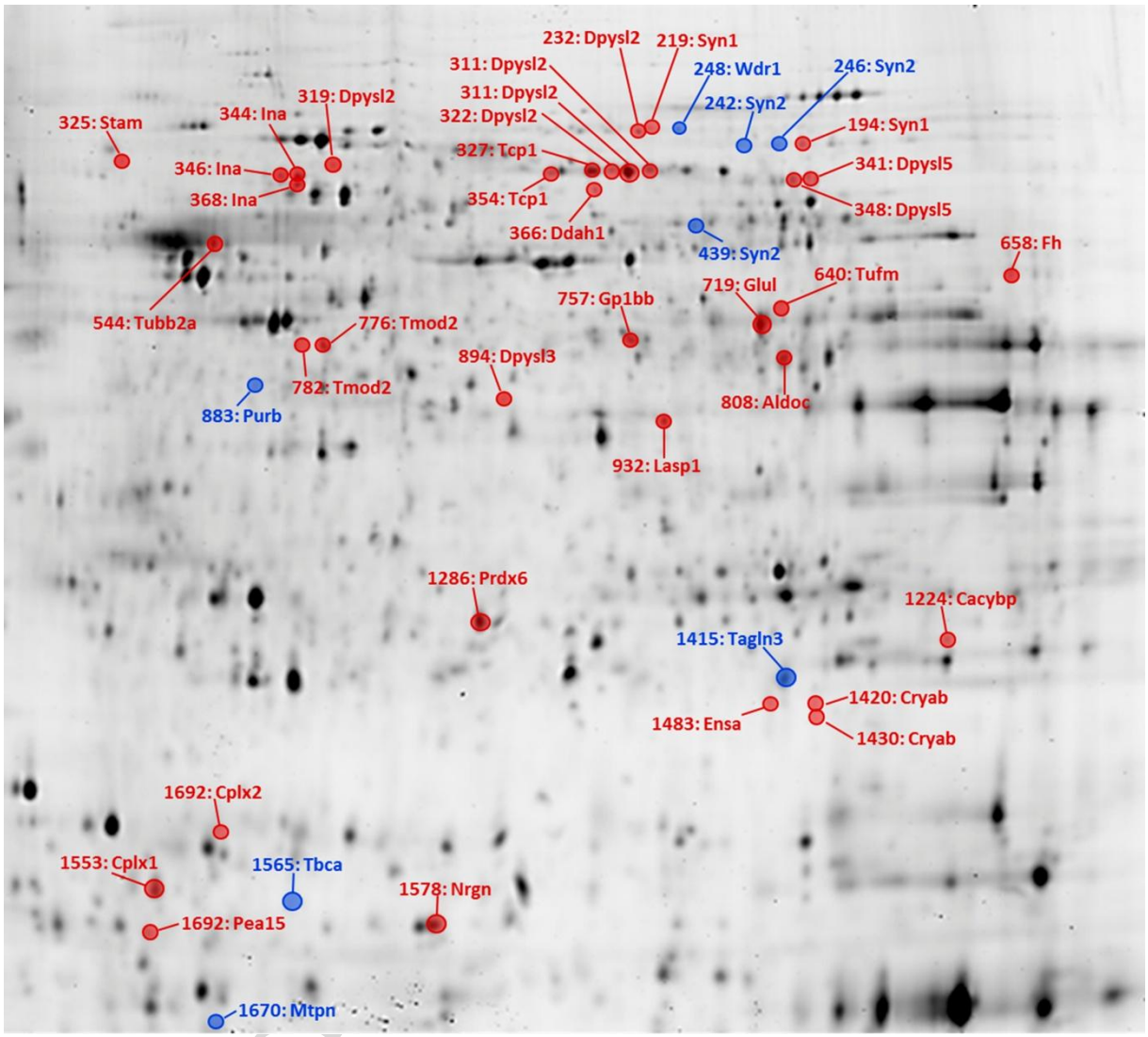


Figure 1



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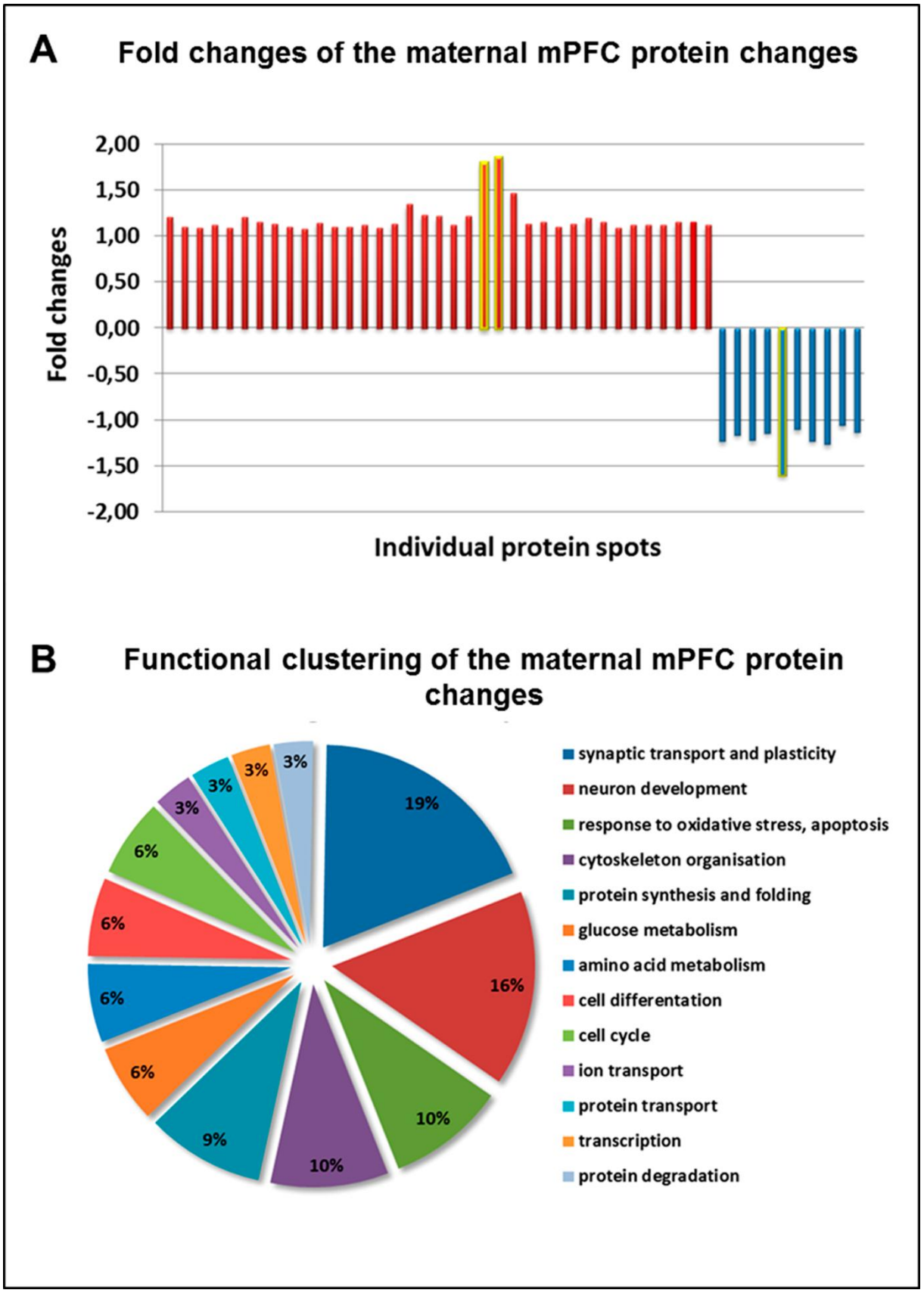
A
Fold changes of the maternal mPFC protein changes
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0,00
-0,50
-1,00
-1,50
-2,00
Fold changes
Individual protein spots
B
Functional clustering of the maternal mPFC protein changes
19%
16%
10%
10%
9%
6%
6%
6%
6%
3%
3%
3%
3%
synaptic transport and plasticity
neuron development
response to oxidative stress, apoptosis
cytoskeleton organisation
protein synthesis and folding
glucose metabolism
amino acid metabolism
cell differentation
cell cycle
ion transport
protein transport
transcription
protein degradation

Figure 3

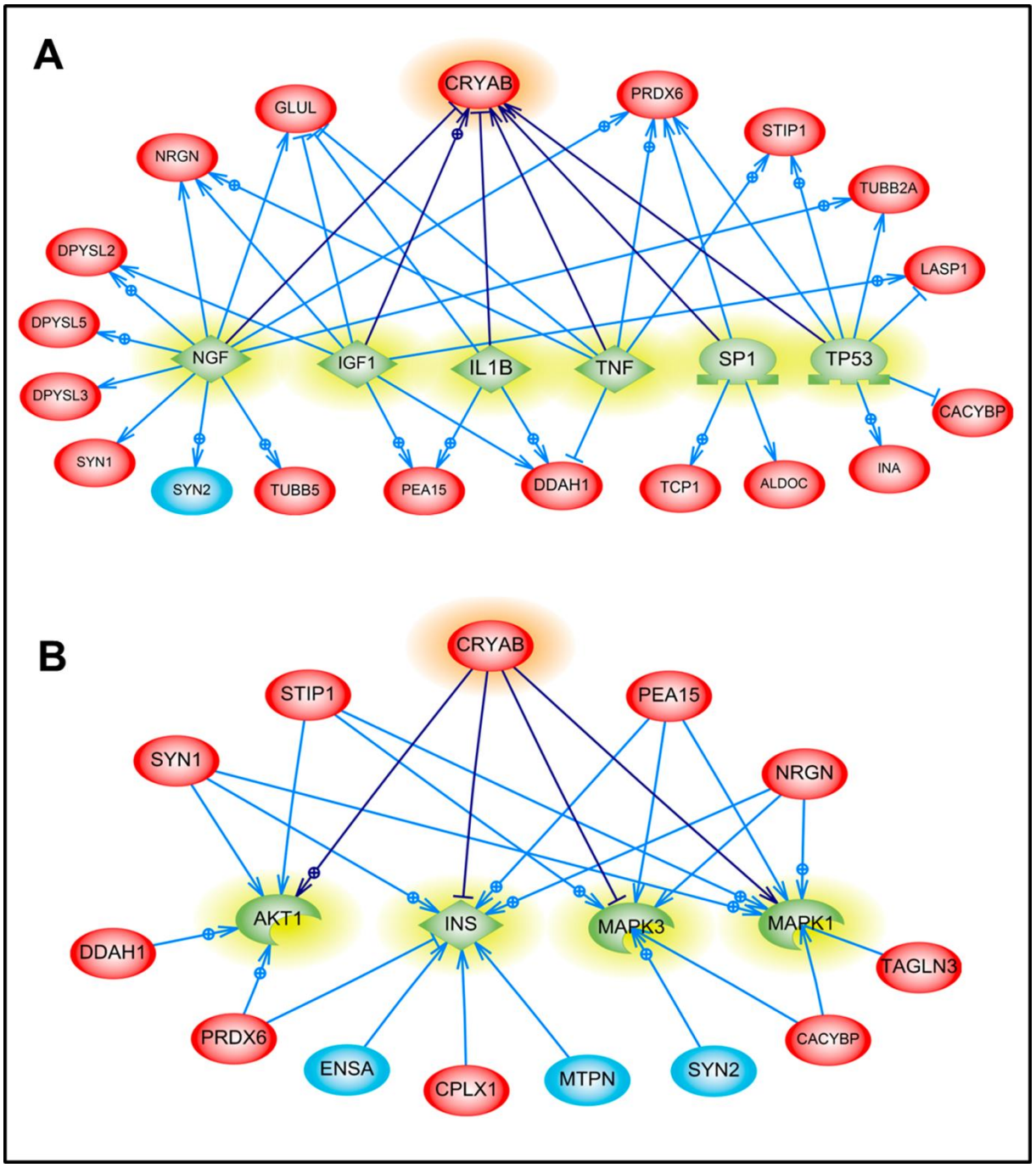


Figure 4

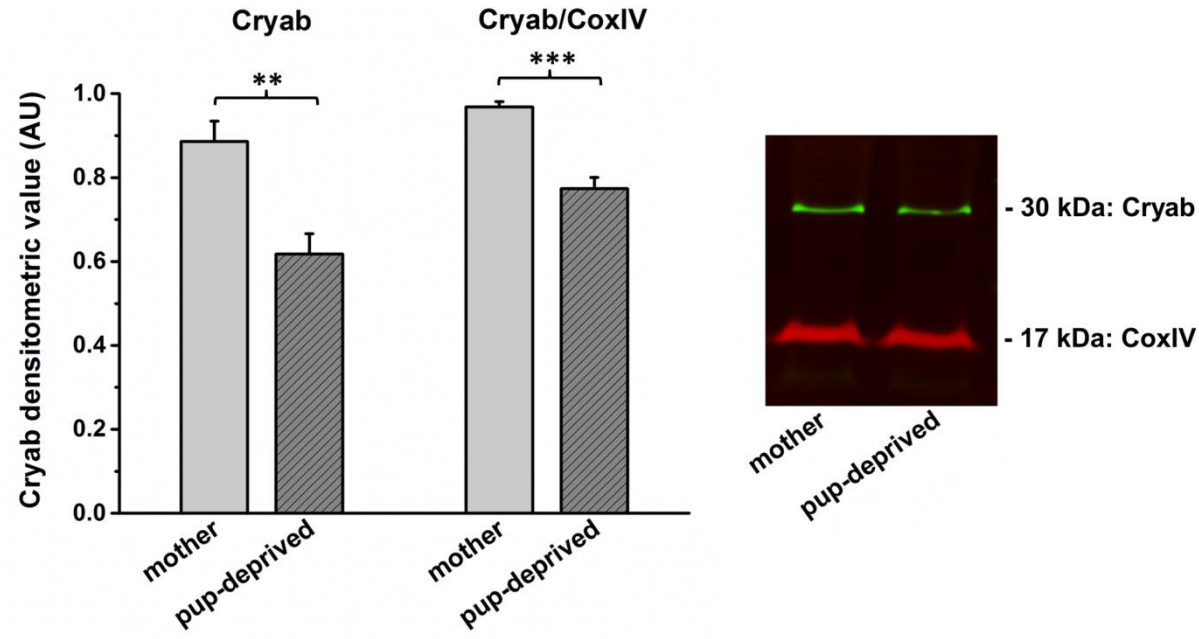


Figure 5

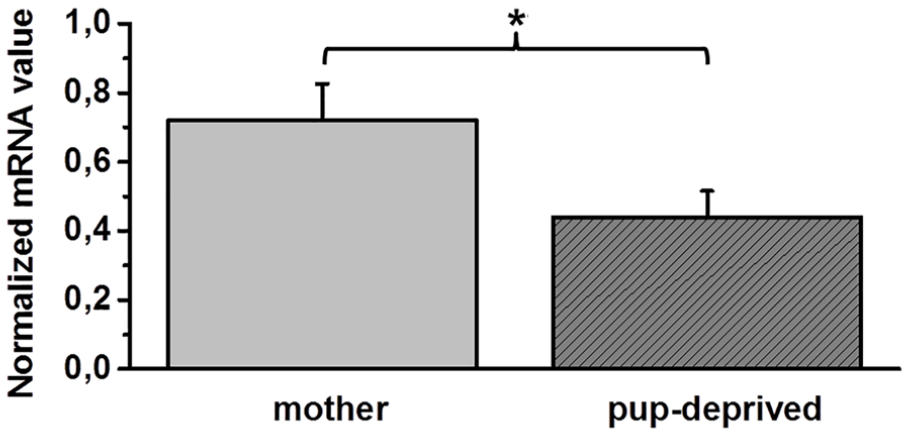


Figure 6

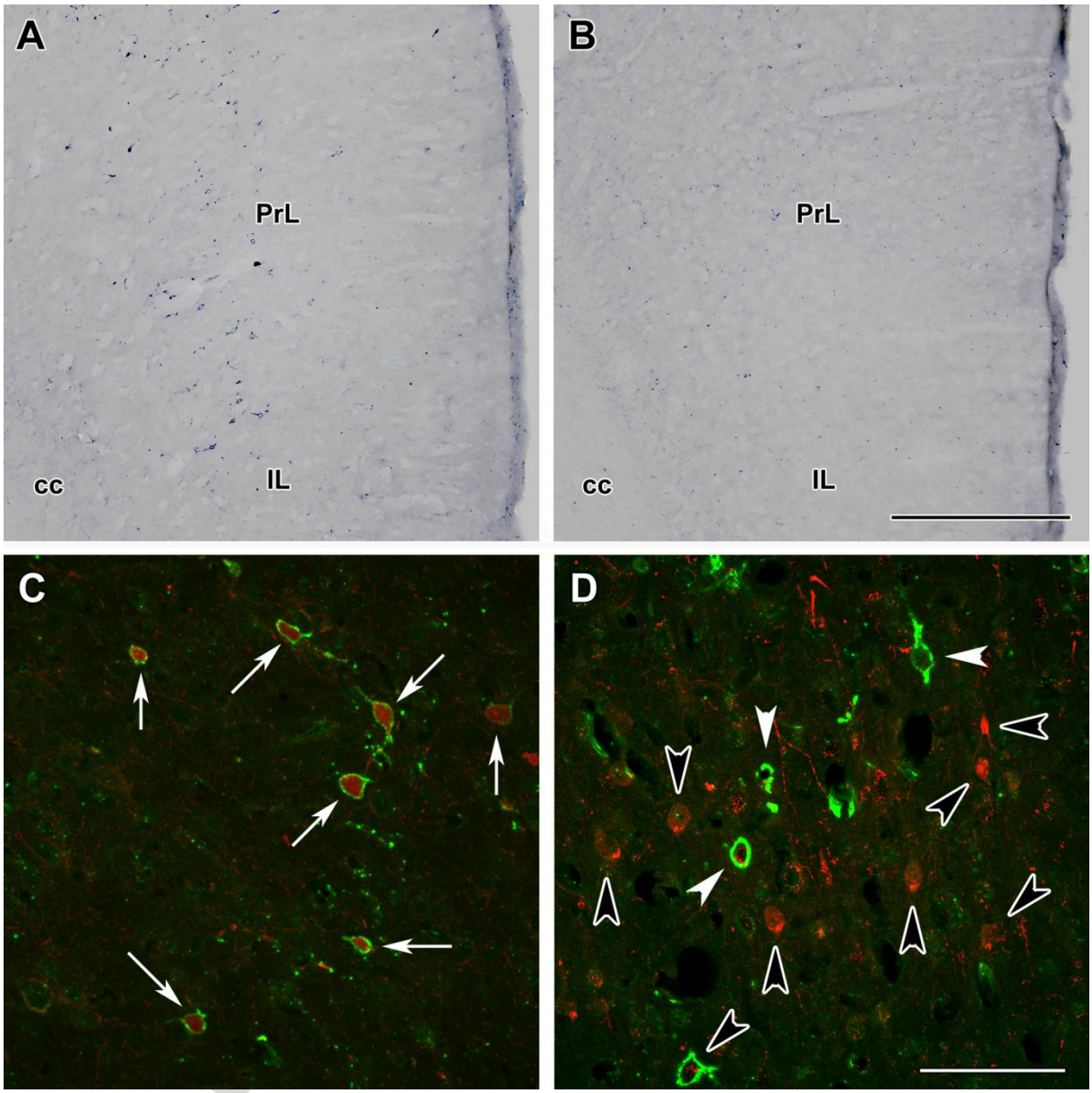
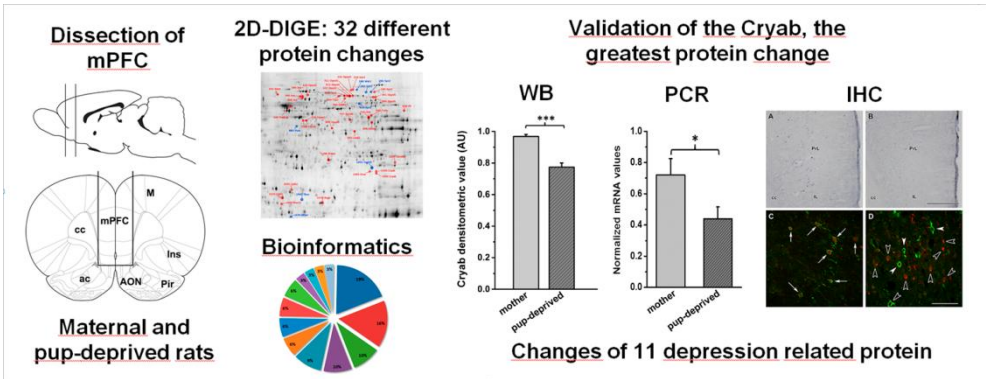


Figure 7



Graphical Abstract

Table 1.

Gene	Forward and reverse primers	Genbank accession number	Position of the PCR product
Alpha-crystallin B chain (Cryab)	ctacagccacttccctgagc acaaacacagcaagcacagg	NM_012935	136-365
Beta actin (Actb)	agggtgtgatggtgggtatg ccagaggcatacagggacaa	NM_031144	200-515
Cytochrome c oxidase subunit IV isoform 1 (Cox4i1)	tctacttcggtgtgccttcg gtgcccttgttcatctcagc	NM_017202	135-379
Glyceraldehyde-3-phosphate-dehydrogenase (Gapdh)	tgccactcagaagactgtgg gtcctcagtgtagcccagga	M17701	540-831
Lactate dehydrogenase A (Ldha)	tgcagcagggtttctatgga ggacttacacactggagcca	NM_017025	1139-1443

Table 2.

MEDIAL PREFRONTAL CORTEX										
ID	**Acc**	**Gene**	**Protein name**	**AR**	***t*-test**	**UP**	**SC%**	**MW(Da)**	**pI**	**Localisation (GO)**
synaptic transport and plasticity										
194	P09951	Syn1	Synapsin-1	1,21	0,0417	15	34,4	73989	9,8	synapse, Golgi
219				1,10	0,0023	17	45,0	73989	9,8	
242	Q63537	Syn2	Synapsin-2	-1,23	0,0075	16	41,1	63457	8,7	synapse
246				-1,16	0,0053	14	41,0	63457	8,7	
439				-1,22	0,0147	13	34,6	63457	8,7	
776	P70566	Tmod2	Tropomodulin-2	1,09	0,0004	19	45,6	39492	5,3	cytoplasm
782				1,12	0,0266	14	41,6	39492	5,3	
1553	P63041	Cplx1	Complexin-1	1,09	0,0298	5	52,2	15122	4,9	cytoplasm, synapse
1578	Q04940	Nrgn	Neurogranin	1,20	0,0499	2	41,0	7496	6,5	cytoplasm, synapse
1692	P84087	Cplx2	Complexin-2	1,15	0,0354	3	38,1	15395	5,1	cytoplasm, synapse
neuron development										
232	P47942	Dpysl2	Dihydropyrimidinase-related protein 2	1,13	0,0143	12	31,5	62278	6,0	cytoplasm, membrane
311				1,10	0,0004	24	62,9	62278	6,0	
319				1,08	0,0192	3	6,5	62278	6,0	
322				1,15	0,0055	38	77,8	62278	6,0	
327				1,10	0,0100	36	68,2	62278	6,0	
301				1,10	0,0017	25	66,3	62278	6,0	
341	Q9JHU0	Dpysl5	Dihydropyrimidinase-related protein 5	1,12	0,0066	19	44,0	61541	6,6	cytoplasm
348				1,09	0,0091	20	54,4	61541	6,6	
894	Q62952	Dpysl3	Dihydropyrimidinase-related protein 3	1,14	0,0016	18	49,1	61968	6,0	cytoplasm
344	P23565	Ina	Alpha-internexin	1,35	0,0198	20	51,1	56116	5,2	neurofilament
346				1,23	0,0441	27	64,0	56116	5,2	
368				1,22	0,0403	31	70,7	56116	5,2	
1394	P37805	Tagln3	Transgelin-3	1,12	0,0159	14	70,9	22501	6,8	nucleus
1415				-1,14	0,0334	7	41,7	22501	6,8	
response to oxidative stress, apoptosis										
1286	O35244	Prdx6	Peroxiredoxin-6	1,22	0,0065	18	78,6	24819	5,6	cytoplasm
1420	P23928	Cryab	Alpha-crystallin B chain	1,80	0,0002	7	34,3	20089	6,8	cytoplasm, nucleus
1430				1,86	0,0059	2	21,7	20089	6,8	
1596	Q5U318	Pea15	Astrocytic phosphoprotein PEA-15	1,47	0,0016	3	30,0	15040	4,9	cytoplasm
cytoskeleton organisation										
1565	Q6PEC1	Tbca	Tubulin-specific chaperone A	-1,60	0,0047	8	43,5	12744	5,4	cytoplasm
544	P85108	Tubb2a	Tubulin beta-2A chain	1,13	0,0281	32	67,0	49907	4,8	cytoplasm, nucleus

545	P69897	Tubb5	Tubulin beta-5 chain	1,15	0,0224	31	66,2	49671	4,8	cytoplasm, nucleus
protein synthesis and folding										
307	O35814	Stip1	Stress-induced-phosphoprotein 1	1,10	0,0020	15	36,6	62571	6,4	cytoplasm, nucleus
317				1,13	0,0095	28	48,3	62571	6,4	
354	P28480	Tcp1	T-complex protein 1 subunit alpha	1,20	0,0030	12	34,0	60360	5,9	cytoplasm
640	P85834	Tufm	Elongation factor Tu, mitochondrial	1,15	0,0066	8	23,9	49523	7,2	mitochondrion
glucose metabolism										
658	P14408	Fh	Fumarate hydratase, mitochondrial	1,09	0,0032	9	24,9	54464	9,1	mitochondrion
808	P09117	Aldoc	Fructose-bisphosphate aldolase C	1,12	0,0002	14	51,0	39284	6,7	cytoplasm, mitochondrion
amino acid metabolism										
366	O08557	Ddah1	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	1,12	0,0041	5	21,4	31426	5,8	cytoplasm, mitochondrion
719	P09606	Glul	Glutamine synthetase	1,12	0,0366	14	41,8	42268	6,6	cytoplasm, mitochondrion
cell differentiation										
1670	P62775	Mtpn	Myotrophin	-1,10	0,0253	7	60,2	12861	5,3	cytoplasm, nucleus
248	Q5RKI0	Wdr1	WD repeat-containing protein 1	-1,23	0,0255	4	16,7	66182	6,1	cytoplasm, cell projection
cell cycle										
757	D3ZDH8	Gp1bb	Septin 5, isoform CRA_d	1,15	0,0354	17	47,4	43893	6,2	synapse, plasma membrane
1483	P60841	Ensa	Alpha-endosulfine	-1,26	0,0476	5	41,3	13335	6,6	cytoplasm
ion transport										
932	Q99MZ8	Lasp1	LIM and SH3 domain protein 1	-1,06	0,0146	3	14,1	29971	6,6	cytoplasm
protein transport										
325	B5DF55	Stam	RCG55706	1,16	0,0065	14	30,3	59593	4,7	cytoplasm
transcription										
883	Q68A21	Purb	Transcriptional activator protein Pur-beta	-1,13	0,0240	6	45,7	33418	5,3	nucleus
protein degradation										
1224	Q6AYK6	Cacybp	Calcyclin-binding protein	1,12	0,0001	8	48,5	26541	7,6	cytoplasm, nucleus

Table 3.

MEDIAL PREFRONTAL CORTEX					
ID	**Acc**	**Gene**	**Protein name**	**AR**	**Protein - depression connection**
synaptic transport and plasticity					
194	**P09951**	**Syn1**	**Synapsin-1**	**1,21**	Increased Syn1 protein level correlated with depression-like behavior **(1)**
219				**1,10**	Syn1 showed differential level of phosphorylation in postmortem dorsolateral prefrontal cortex tissues from major depressive disorder patients **(2)**
1553	**P63041**	**Cplx1**	**Complexin-1**	**1,09**	Cplx1 showed decreased mRNA level in the hippocampus from postmortem bipolar disorder brain **(3)**
					Cplx1 showed decreased expression after antidepressant paroxetine exposure in rat hippocampus **(4)**
1692	**P84087**	**Cplx2**	**Complexin-2**	**1,15**	Cplx2 showed decreased protein level in the anterior cingulate cortex from postmortem major depression brain **(5)**
					Downregulation of Cplx2 correlated with depression-like behaviour **(6)**
1578	**Q04940**	**Nrgn**	**Neurogranin**	**1,20**	Altered Nrgn phosphorylation and decreased protein level in hippocampus and prefrontal cortex are associated with depression-like behaviors in rats following forced swim stress **(7)**
neuron development					
232	**P47942**	**Dpysl2**	**Dihydropyrimidinase-related protein 2**	**1,13**	Dpysl2 showed decreased protein level in the prefrontal cortex of a rat model of depression **(8)**
311				**1,10**	
319				**1,08**	Dpysl2 showed increased protein level in learned helpless rat depression model **(9)**
322				**1,15**	After antidepressant drugs Dpysl2 showed increased level in rat hippocampus **(10)**
327				**1,10**	Dpysl2 showed differential level of phosphorylation and decreased level of expression in postmortem dorsolateral prefrontal cortex tissues from major depressive disorder patients **(2)**
301				**1,10**	Dpysl2 protein level decreased in female rat hippocampus following exposure to a terrified sound stress that is a risk factor of depression **(11)**
344	**P23565**	**Ina**	**Alpha-internexin**	**1,35**	Ina protein level decreased in female rat hippocampus following exposure to a terrified sound stress that is a risk factor of depression **(11)**
346				**1,23**	
368				**1,22**	
response to oxidative stress, apoptosis					
1286	**O35244**	**Prdx6**	**Peroxiredoxin-6**	**1,22**	After antidepressant paroxetine exposure Prdx6 showed decreased protein level in rat hippocampus **(12)**
1420	**P23928**	**Cryab**	**Alpha-crystallin B chain**	**1,80**	Cryab showed differential level of phosphorylation in postmortem dorsolateral prefrontal cortex tissues from major depressive disorder patients **(2)**
1430				**1,86**	
1596	**Q5U318**	**Pea15**	**Astrocytic phosphoprotein PEA-15**	**1,47**	Pea-15 protein level increased in antidepressant-treated prefrontal cortex of major depression postmortem brains **(13)**
amino acid metabolism					

366	**O08557**	**Ddah1**	**N(G),N(G)-dimethylarginine dimethylaminohydrolase 1**	**1,12**	Decreased Ddah1 protein level correlated with magnesium restriction-induced depression like behavior **(14)**
719	**P09606**	**Glul**	**Glutamine synthetase**	**1,12**	Glul showed decreased expression level in cerebral cortex from major depressive disorder patients **(15)**
protein transport					
325	**B5DF55**	**Stam**	**RCG55706**	**1,16**	Stam showed decreased protein level in mice hippocampus after exposure to prenatal stress that is a risk factor of depression **(17)**
ion transport					
932	**Q99MZ8**	**Lasp1**	**LIM and SH3 domain protein 1**	**-1,06**	Decreased hippocampal Lasp1 protein level correlated with depression-like behavior **(16)**

CONFLICT OF INTEREST STATEMENT

The authors of the manuscript have no conflict of interest.

Biological significance

The behavior and emotional state of females change robustly when they become mothers. The brain, which governs these changes, may also undergo molecular alterations in mothers. As no proteomics approaches have been applied regarding maternal changes in the brain, we addressed this issue in the mPFC as this brain area is the uppermost cortical center of maternal control and the associated mood changes. The high number of protein-level alterations found between mothers taking care of their litter and those without pups indicates that pup nursing is associated with cortical protein-level changes. Alterations in proteins participating in synaptic transport, plasticity and neuron development suggest neuroplastic changes in the maternal brain. In turn, the relatively high number of altered proteins in the mPFC associated with depression suggests that the physiological effects of the protein-level alterations in the maternal mPFC could promote the incidence of postpartum depression. Alpha-crystallin B chain, a protein confirmed to be increased during maternal behaviors, was selectively found in parvalbumin cells, which, as fast-spiking interneurons, are associated with depression. The function of alpha-crystallin B chain should be further investigated to establish whether it can be used to identify drug targets for future drug development.

Highlights

We identified 32 protein changes in the medial prefrontal cortex (mPFC) of rat dams.

The most abundant clusters are: synaptic transport and plasticity, neuron development.

Common regulators are growth factors and cytokines, common target the MAP kinases.

A number of proteins altered in the mPFC are associated with depression.

Increase of alpha-crystallin B chain was confirmed, and shown in parvalbumin cells.