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Proteomic characterization in the hippocampus of prenatally stressed rats

J. Mairesse[a], A.S. Vercoutter-Edouart[b], J. Marrocco[a], A.R. Zuena[c], A. Giovine[a,c], F. Nicoletti[c,d], J.C. Michalski[b], S. Maccari[a], S. Morley-Fletcher[a,*]

[a]Neural Plasticity Team-UMR CNRS/USTL n° 8576 Structural and Functional Glycobiology Unit, North University of Lille, France
[b]Cell Signaling Team-UMR CNRS/USTL n° 8576 Structural and Functional Glycobiology Unit, North University of Lille, France
[c]Dept. of Pharmacology, Sapienza University of Rome, Italy
[d]I.N.M. Neuromed, Pozzilli, Italy

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ABSTRACT

Rats exposed to early life stress are considered as a valuable model for the study of epigenetic programming leading to mood disorders and anxiety in the adult life. Rats submitted to prenatal restraint stress (PRS) are characterized by an anxious/depressive phenotype associated with neuroadaptive changes in the hippocampus. We used the model of PRS to identify proteins that are specifically affected by early life stress. We therefore performed a proteomic analysis in the hippocampus of adult male PRS rats. We found that PRS induced changes in the expression profile of a number of proteins, involved in the regulation of signal transduction, synaptic vesicles, protein synthesis, cytoskeleton dynamics, and energetic metabolism. Immunoblot analysis showed significant changes in the expression of proteins, such as LASP-1, fascin, and prohibitin, which may lie at the core of the developmental programming triggered by early life stress.

1. Introduction

The low discordance of depression between monozygotic twins and the slow progress in identifying genetic risk factors suggest that epigenetic changes largely contribute to the individuals' vulnerability to major depressive disorder [1]. Both human and animal studies suggest that exposure to stressful events during critical periods of brain development triggers an epigenetic programming leading to low resilience to stress in the adult life [2–8]. Abnormalities of synaptic transmission and plasticity in the hippocampus represent an integral part of this epigenetic program. For example, early life stress resulting from low maternal care in rodents causes a permanent reduction in the length of dendritic branching and the number of dendritic spines associated with an impairment of synaptogenesis and long-term potentiation in the hippocampus [9–11]. This fits nicely with the clinical evidence that poor parental care can compromise cognitive development [12,13].

Rats exposed to prenatal restraint stress (PRS) develop long-lasting biochemical and behavioral changes that likely reflect the induction of a pathological epigenetic programming [14,15], and therefore represent a model that meets the criterium of *construct validity* because it replicates environmental factors implicated in the etiology of depression and other stress-related disorders [1]. Alterations induced by PRS comprise a dysfunction of the hypothalamo-pituitary-adrenal (HPA) axis which is reversed by cross fostering at birth [16], a generalized disorganization of circadian rhythms and the sleep–wake cycle, an age-dependent impairment in spatial

* *Corresponding author at:* North University of Lille, Neuroplasticity Team, CNRS UMR 8576/ UGSF, Structural and Functional Glycobiology Unit, Bât C9, Avenue Mendeleiev, 59655 Villeneuve d'Ascq France. Tel.: +33 32033 6042; fax: +33 32043 6555.
E-mail address: sara.morley-fletcher@univ-lille1.fr (S. Morley-Fletcher).

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learning, a lifelong reduction of hippocampal neurogenesis, and changes in the levels of brain-derived neurotrophic factor (BDNF), cyclic-AMP responsive element binding protein (CREB), and group-I and group-II metabotropic glutamate receptors in the hippocampus [16–20]. Remarkably, some of these changes are reversed by chronic antidepressant treatment [21–23]. Hence, the rat model of PRS is particularly valuable for a systematic analysis of hippocampal proteins that are the product of the epigenetic programming leading to a low resilience to stress and to an anxious/depressive phenotype in the adult life. Here, we examined the protein expression profile in the hippocampus of adult rats exposed to PRS by using a proteomic approach based on the use of two-dimensional electrophoresis coupled with mass spectrometry.

2. Materials and methods

2.1. Animals

Nulliparous female Sprague–Dawley rats, weighing approximately 250 g, were purchased from a commercial breeder (Harlan). Animals were housed at constant temperature (22±2 °C) and under a regular 12 h light/dark cycle (lights on at 8.00 a.m.). Pregnant females were randomly assigned to stressed or control groups (n=12 per group).

2.2. Stress protocol

Animals were subjected to PRS according to our standard protocol [16,21]. From day 11 of pregnancy until delivery, pregnant female rats were subjected to three stress sessions daily (45 min each), during which they were placed in transparent plastic cylinders and exposed to bright light. Only male offspring from litters containing 10–14 pups with a comparable number of males and females were used for the experiments. All experiments followed the rules of the European Communities Council Directive 86/609/EEC. The prenatal stress procedure was approved by the local ethical committee.

2.3. Protein sample preparation and 2D analysis in the hippocampus

Hippocampi of PRS and control rats (n=6/group) were rapidly dissected, frozen on dry ice and stored at −80 °C. Samples were then homogenized with a glass/Teflon homogenizer at a concentration of 10% (w/v) in a solubilizing solution containing: 7 M urea (Sigma-Aldrich, St. Louis, MO, USA), 2 M thiourea (Fluka, Buchs, Switzerland), 40 mM Tris (Sigma-Aldrich), 2% CHAPS (Fluka), and Complete™ protease inhibitor (Roche, Basel, Switzerland). Samples were sonicated three times for 10 s on ice with an ultrasonic processor with probe (Ultrasonic 2000, Dynatech Laboratories Inc., Chantilly, VA, USA). The extract was centrifuged at 1000 *g* and the pellet discarded. An aliquot of this supernatant was used to measure protein concentration by the Bradford method [24]. 100 μg of proteins was separated by 2D electrophoresis following a step of passive rehydration on 18 cm immobilized pH gradient strips (IPG; non-linear pH gradient of 3–10, GE Healthcare, France) overnight. Focusing was carried out for 24 h at 20 °C for a total of 100,000 Vh on a pHaser isoelectric focusing system (Genomic solutions, Cambridgeshire, UK). The focused IPG strips were equilibrated for 20 min with gentle shaking in an equilibration solution (6 M urea, 2% SDS, 375 mM Tris pH 8.8, 30% glycerol) containing 1% DTT, and then with 2% iodoacetamide. The strips were applied to 10% SDS polyacrylamide gels using the Investigator System (Genomic Solutions), and finally, 2D gels were silver-stained. For each animal, 2-D gel electrophoresis was performed in triplicates for a total of 36 gels.

Electrophoresis images of gels were digitized using the GS-710 densitometer system (Bio-Rad). 2D gel analysis was carried out with Progenesis SameSpots software (Nonlinear Dynamics, Ltd).

2.4. Statistics

The aligned images were grouped into their corresponding PRS or control group and the statistically ranked list of spots was evaluated in the review stage of the SameSpots software. Protein levels were evaluated as volumes (spot area×optical density) for the protein spots matched among gels. Spot volume for valid spots was normalized to total density for each gel. Our criteria for evaluation of protein spots were based on an ANOVA p-value<0.05 as calculated with the built-in statistical tools in the software and a minimum of 1.5-fold intensity (normalized volume) in protein content between PRS and control animals. Then, only spots within the range of 1.5–3.5 fold change were cut out (24 spots) and processed for LC–MS/MS analysis.

2.5. Protein identification with LC–MS/MS

The gel with the highest spot intensity was selected for manual excision for evaluation by mass spectrometry. Spots of interest were carefully cut from the gel, destained in a solution containing 1.6% thiosulfate and 1% potassium ferricyanide, extensively washed in water, and then submitted to *in-gel* trypsin digestion. Briefly, after reduction and alkylation, trypsin digestion was performed overnight at 37 °C in 25 mM ammonium bicarbonate (porcine mass spectrometry grade MSG-Trypsin; G-Biosciences, Agro-Bio, La Ferté St Aubin, France). Peptides were extracted in 45% acetonitrile/45% water/10% trifluoroacetic acid (TFA) (v/v/v) and then dried in a speed-vac (Eppendorf) before nano-high pressure liquid chromatography (HPLC)–MS/MS analysis. NanoLC-NanoESI–MS/MS analyses were performed either on an ion trap mass spectrometer (LCQ Deca XP+, Thermoelectron, San Jose, CA) equipped with a nano-electrospray ion source coupled to a nano-flow high-pressure liquid chromatography system (LC Packings Dionex, Amsterdam, The Netherlands) as previously described [25], or on an hybrid quadrupole time-of-flight mass spectrometer (Q-Star, Applied Biosystems, Foster City, California, USA) equipped with a nano-electrospray ion source coupled with a nano HPLC system (LC Packings Dionex, Amsterdam, The Netherlands). Peptidic samples were dissolved in 5 μL 95% H2O/5% ACN / 0.1% HCOOH (v/v/v) (solvent A) and were injected into the mass spectrometer using the Famos auto-sampler (LC Packings Dionex, Amsterdam, The Netherlands). Samples were desalted and concentrated on a reserved-phase precolumn of 0.3 mm i.d.×5 mm (Dionex) by solvent A delivered by the Switchos

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pumping device (LC Packings Dionex), at a flow rate of 10 μL/min for 3 min. Peptides were then separated on a 75 μm i.d.×15 cm C18 Pepmap column (Dionex). The flow rate was set at 200 nL/min. Peptides were eluted using a 0% to 35% linear gradient of solvent B (25% H2O/75% ACN/0.1% HCOOH) in 80 min then a 35% to 100% linear gradient of solvent B in 10 min and finally 100% of solvent B was maintained for 5 min. Coated electrospray needles were obtained from New Objective (Woburn, Massachusetts, USA). The spray voltage was 1.65 kV. The mass spectrometer was operated in the positive ion mode. Data acquisition was performed in a data-dependent mode consisting of, alternatively, a full-scan MS over the range *m*/z 300–2000, and a full-scan MS/MS of the ion selected over the range *m*/z 50–2000 in an exclusion dynamic mode (the most intense ion is selected and excluded for further selection for a duration of 30 s). MS/MS data were acquired using a mass tolerance of 50 mmu and the collision energy was automatically fixed by the device. For the automated database search of fragment ion spectra, the Analyst QS software and Mascot dll script were used and final database searching was performed using Mascot software (Matrix Science London, UK, MS/MS ion search module), in the Swiss-Prot database (Sprot 0411, 525,207 sequences). Search parameters were as follows: Rattus as the taxonomic category, 100 ppm tolerance for the parent ion mass and 50 mmu for the MS/MS fragment ions, one missed cleavage allowed, carbamidomethylcysteine as a fixed modification, and methionine oxidation as a possible modification. Only proteins with a significant Mascot score were taken into consideration and reported after manual verification of the fragmentation spectra.

2.5. *Western blot validation of identified proteins*

A separate set of animals was used for immunoblotting experiments. Four to six animals per group were analyzed in duplicate. Rats were killed by decapitation and brains rapidly removed; hippocampi (dorsal and ventral where described) were dissected and stored at −80 °C until homogenization.

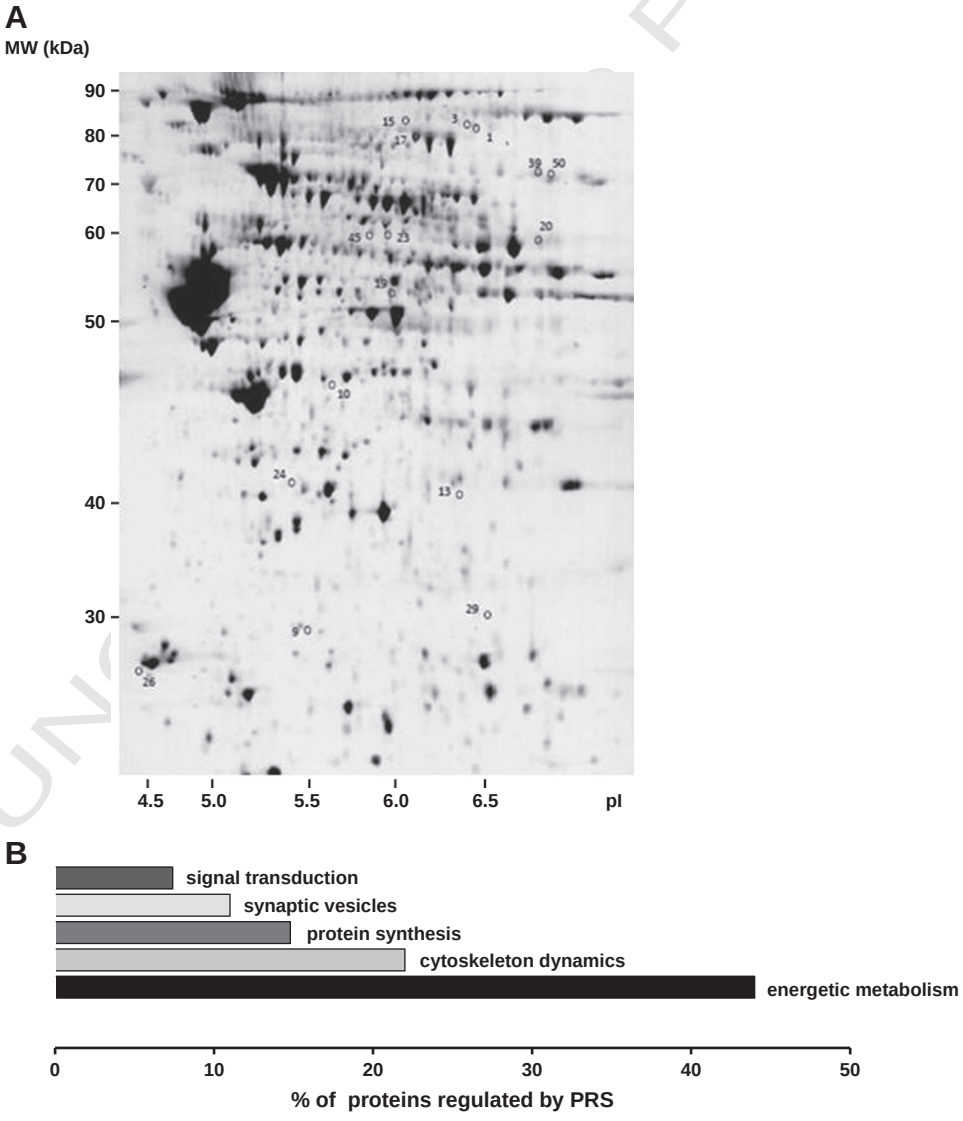

Fig. 1 – (A) Representative 2D gel image with spots of proteins listed in Table 1 in the 3–10 pH range. (B) Functional clustering of the identified proteins regulated by PRS in the adult hippocampus.

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Tissues were homogenized at 4 °C with a TissueRuptor (Quiagen) in lysis buffer (pH 7.4) containing: 320 mM Sucrose, 5 mM Hepes, 500 mM sodium fluoride, 10% SDS and phosphatase/protease inhibitor (Sigma). BCA assay was used to determine protein concentration. Lysates were resuspended in laemli reducing buffer and 25 μg of each sample was first separated by electrophoresis on 8–12% SDS-polyacrylamide gels and suddenly later transferred to nitrocellulose membranes (Biorad). Transferring was performed at 4 °C in a buffer containing 35 mM TRIS, 192 mM glycine and 20% methanol.

The following primary antibodies were used to detect the relevant proteins: anti-Prohibitin (Thermo Scientific; 1:1000), anti-LASP-1 (Millipore; 1:1000), anti-Fascin (Santa Cruz; 1:2000), anti-Transferrin (AbCam; 1:5000), anti-β-Actin (Sigma; 1:80000). Secondary antibodies directed against rabbit or chicken were used at 1:10,000 dilution. Densitometric analysis was performed with Quantity One software (Bio-Rad) associated to a GS-800 scanner. A ratio of target to β-Actin was determined and these values were compared for statistical significance with the Student's t-test.

3. Results

3.1. *PRS altered the hippocampal proteome*

To identify novel proteins modified by PRS we compared the proteome in the hippocampus of adult male PRS and control rats. Analysis of two-dimensional electrophoresis patterns by using Progenesis SameSpots Software revealed that the densities of 24 spots were significantly different ($p<0.05$) between control and PRS rats.

Among them, 18 spots with a 1.5–3.5 fold change shown on a gel in Fig. 1A, were unambiguously identified as known proteins by nanoLC–tandem mass spectrometry. A total of 26 different identified proteins were thus sorted into the following 5 groups based on their biological function: (i) signal transduction; (ii) synaptic vesicles; (iii); protein synthesis (iv) cytoskeleton dynamics; and (v) energetic metabolism (Fig. 1B). These proteins are listed in Table 1 and Supplemental Table 1. We identified up to 6 different proteins per regulated spot in some cases, due to possible overlapping protein spots in the wide pH 3–10 range. The image analysis identified spots 3 and 9 as being up-regulated by PRS whereas all the other spots were found to be down regulated. We could identify mainly soluble and cytosolic proteins. It is therefore likely that many other changes remained undetected, particularly those involving low abundant proteins, or more hydrophobic and high molecular weight proteins.

3.2. *Immunoblotting validation of proteomic data*

In order to confirm proteomic data in the hippocampus, the expression of proteins previously identified within the regulated spot were examined by immunoblot analysis in a separate set of animals (Fig. 2). We found that PRS decreased the expression of Lasp-1 (spot no. 13; $F(1,8)=7.73$, $p<0.05$) and increased the expression of transferrin (spot no. 3; $F(1,8)=10.21$, $p<0.05$), prohibitin (spot no. 9; $F(1,8)=13.19$, $p<0.05$), and fascin (spot no. 19; $F(1,8)=6.16$, $p<0.05$). The increase in the expression of fascin could appear discordant with the general down regulation profile of spot 19, as revealed by MS/MS analysis. However, fascin accounts for the 30% only of the peptides mixture if we take into account the fifteen different sequences of peptides identified within the spot. The decrease in intensity of spot 19 observed in the comparative analysis of 2D-gels may thus come from the other identified candidates.

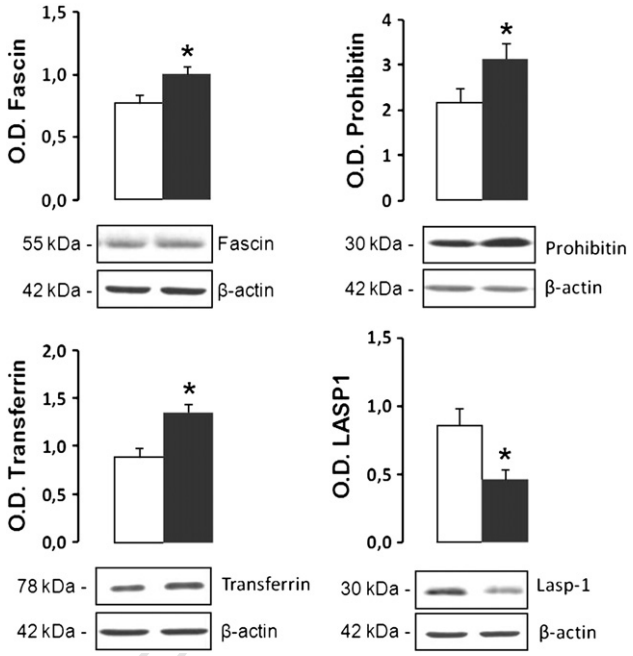

Fig. 2 – Immunoblot analysis of fascin, prohibitin, transferrin, and LASP-1 in the hippocampus of control and PRS adult rats. Values are means+S.E.M. of 6 biological replicates. *$p<0.05$ vs. controls.

4. Discussion

This study applied for the first time a proteomic approach to the rat model of PRS that recapitulates some of the features of stress-related disorders in humans [14,15]. This model is valuable for the study of the pathological epigenetic programming induced by stressful events occurring early in life (see Introduction and references therein). We found that PRS altered the expression profile of several hippocampal proteins, including proteins involved in signal transduction, intracellular trafficking and membrane fusion of synaptic vesicles. Interestingly, some of the proteins modified by PRS such as synapsin 2, LASP 1 and prohibitin, are known to be glucocorticoid regulated. This is relevant because PRS rats present an increased secretion of glucocorticoid in response to stress [16]. Indeed, inactivation of glucocorticoid receptor in the hippocampus reduces levels of synapsins in mice (2-), and acute corticosterone treatment enhances Lim family proteins [27], among which there is LASP1 , a dynamic focal adhesion protein involved in mechanisms of cell migration and survival [28,29]. Prohibitin also was modified by PRS. Such protein is a membrane-bound chaperone which inhibits DNA synthesis and has been implicated in aging, mitochondrial inheritance

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Table 1 – Selected list of proteins whose expression was modified in the hippocampus of PRS rats. Proteins were separated by 2D electrophoresis, and nano-LC–MS/MS analysis was performed after trypsin digestion on silver-stained spots. The biological function of the identified proteins is indicated based on gene ontology. Accession number, entry name, and theoretical MW and pI are indicated, according to the UniProtKB database on the Expasy server. For each candidate, the Mascot score and the number of matched peptides obtained from the MS/MS ion Search module (Mascot) are indicated. Spot numbers are reported according to Fig. 1A. (*) Synapsin-2 and vesicle-fusing ATPase proteins have been identified in spot nos. 39 and 50 (see Suppl Table 1). The higher score obtained for each of these candidates is reported here.

Spot	Biological process	Protein name	Entry name	Accession	MW Kda	pI	Mascot score	Peptides
	Signal transduction							
19		CaMK2	KCC2A	P11275	54	6.6	62	1
13		Phytanoyl CoA hydroxylase interacting protein	PHYYIP	Q568Z9	38	6.5	134	2
	Synaptic vesicles							
39		Synapsin-2(*)	SYN2	Q63537	63	8.73	78	1
26		Synaptosomal-associated protein 25	SNAP25	P60881	23	4.66	235	8
3		Syntaxin binding protein 1	STXB1	P61765	68	6.49	161	4
	Protein synthesis							
9		Prohibitin	PHB	P67779	29	5.5	191	5
50		Elongation factor 1-alpha 1	EF1A1	P62630	50	9.10	67	1
19		T-complex protein1 beta subunit	TCPB	Q5XIM9	57	6.01	89	2
19		F-box/LRR-repeat protein 16	FXL16	Q5MJ12	52	6.1	147	3
	Cytoskeleton dynamics							
19		Fascin	FSCN_1	P85845	55	5.8	169	5
13		LASP-1	LASP1	Q99MZ8	30	6.5	64	2
15		Dihydropyriminidase-like2	DPYL2	P47942	62	5.9	321	8
24		Guanine nucleotide-binding protein G(olf) subunit alpha	GNAL	P38406	45	6.23	39	1
50		Vesicle-fusing ATPase(*)	NSF	Q9QUL6	83	6.55	51	3
17		Mitochondrial import receptor subunit TOM70	TOM70	Q75Q39	68	7.4	117	3
	Energetic metabolism							
3		Transferrin	TRFE	P12346	78	7.14	121	3
10		Phosphomannose isomerase	PMI	Q68FX1	47	5.7	187	4
1		6-phosphofructokinase type C	K6PP	P47860	86	6.95	175	5
10		Adenosine kinase	ADK	Q64640	40	5.7	66	2
29		ATP synthase subunit gamma, mitochondrial	ATPG	P35435	30	8.87	37	2
23		Glucose-6-phosphate 1-dehydrogenase	G6PD	P05370	60	5.97	64	3
24		Isocitrate dehydrogenase [NAD] subunit alpha	IDH3A	Q99NA5	40	6.47	247	7
29		Nitrilase homolog 2	NIT2	Q497B0	31	6.9	41	2
20		Pyruvate kinase isozymes M1/M2	KPYM	P11980	58	6.63	521	18
19		Succinate-semialdehyde-deydrogenase	SSDH	P51650	56	8.3	97	2
19		Tryptophanyl-tRNA-ligase	SYWC	Q6P7B0	54	6.0	145	2

and apoptosis (for review see Ref. 30). The increment of prohibitin induced by PRS is in line with other reports about increased probitin levels after glucocorticoids exposure during early postnatal life such as maternal separation [31] or chronic stress (restraint) in adult life [32]. Thus, we provide the first evidence that *in utero* exposure to stress persistently affects the expression in the hippocampus of proteins from different functional categories, which are known to be regulated by stress and/or glucocorticoids. This observation underlines the putative involvement of the early exposure to glucocorticoids in the permanent modification of the hippocampal proteome in the PRS model.

PRS also increased expression of Fascin, an actin-bundling protein that lies downstream of the GTP-binding protein, Rab35, in the regulation of cytoskeleton dynamics and formation of filopodia and growth cones [33,34]. The fascin-encoding gene, FSCN1, is positively regulated by CREB and is induced during neuronal differentiation of NT2 precursor cells [35]. In addition, fascin is up-regulated in neuroectodermal spheres derived from human embryonic stem cells, and is highly expressed in the subventricular zone of the fetal mouse brain [36]. These data suggest that fascin coordinates cytoskeletal changes associated with neuronal differentiation, although the precise role of this protein in the adult hippocampal neurogenesis remains to be determined. PRS rats showed an increased expression of fascin in spite of the observed reduction of phospho-CREB levels and neurogenesis in the hippocampus [18,20,23]. In contrast, fascin is down-regulated in the ventral hippocampus of normal rats treated with the antidepressant, escitalopram [30]. Perhaps fascin acts as a negative regulator of adult neurogenesis and antidepressants enhance neurogenesis by reducing the expression of fascin. This interesting hypothesis warrants further investigation.

A number of proteins involved in cellular metabolism were modified by PRS. One example was phosphomannose isomerase, a key enzyme in the biosynthetic pathway of N-glycosylprotein [37]. Protein glycosylation critically regulates different aspects of neuronal function including synaptic plasticity [38], and has been implicated in the pathophysiology of neurodegenerative

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disorders [39,40]. However, inactivating mutations of phosphomannose isomerase causes the congenital disorder of glycosylation type Ib, in which the CNS is not affected [41,42]. Thus, the precise relationship between phosphomannose isomerase and the pathological phenotype of PRS rats remains to be determined. Other identified proteins were 6-phosphofructokinase, pyruvate kinase and glucose-6-phosphate dehydrogenase, three enzymes involved in glucose utilization and energetic metabolism that are activated by insulin [43,44]. Changes in the expression of the three enzymes could contribute to the development of insulin resistance and altered glucose metabolism seen in PRS rats [45,46]. A decreased energetic metabolism is expected in light of the depressive phenotype and the negative resilience to stress exhibited by PRS rats [14,15,47].

In conclusion, our data offer the first evidence that PRS induces long-lasting changes in the expression profile of hippocampal proteins that likely reflect a pathological epigenetic program triggered in the perinatal life. Anxiety generated by restraint stress in pregnant mothers [48] might influence brain development during the fetal life as a result of malnutrition or excessive exposure to maternal corticosteroids [49]. Alternatively, the epigenetic misprogramming of PRS rats can be the consequence of the low maternal care in the first week of postnatal life induced by gestational stress (personal observations from the laboratory). The latter hypothesis is more likely because there is compelling evidence that low maternal care causes permanent changes in gene function and behavior in the offspring [2,7,50], and cross-fostering, which increased maternal care, prevents at least the abnormal HPA response to stress induced by PRS in particular on MR and GR hippocampal receptors [16]. Changes in hippocampal proteins seen in PRS rats may facilitate the identification of novel molecular processes and candidate genes involved in the regulation of the stress response and in the pathophysiology of mood disorders.

5. Uncited reference

[26]

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2011.12.017.

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