Phosphoproteomic analysis of the striatum from pleiotrophin knockout and midkine knockout mice treated with cocaine reveals regulation of oxidative stress-related proteins potentially underlying cocaine-induced neurotoxicity and neurodegeneration.

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Abbreviations

5'(3')-deoxyribonucleotidase (NT5C), endoplasmic reticulum resident protein 29 (ERP29), endoplasmic reticulum resident protein 60 (ERP60), extracellular signal-regulated kinase (ERK), glutamate dehydrogenase 1 (GLUD1), hemoglobin subunit beta-1 (HBB1), hemoglobin subunit beta-2 (HBB2), immobilized metal affinity chromatography (IMAC), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), midkine (MK), MK-/- cocaine (MC), MK-/- saline (MS), Parkinson's disease (PD), peroxiredoxin-6 (PRDX6), pleiotrophin (PTN), PTN-/- cocaine (PC), PTN-/- saline (PS), receptor protein tyrosine phosphatase (RPTP), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), wild type (WT), WT cocaine (WC), WT saline (WS).

ABSTRACT

The neurotrophic factors pleiotrophin (PTN) and midkine (MK) are highly upregulated in different brain areas relevant to drug addiction after administrations of different drugs of abuse, including psychostimulants. We have previously demonstrated that PTN and MK modulate amphetamine-induced neurotoxicity and that PTN prevents cocaine-induced cytotoxicity in NG108-15 and PC12 cells. In an effort to dissect the different mechanisms of action triggered by PTN and MK to exert their protective roles against psychostimulant neurotoxicity, we have now used a proteomic approach to study protein phosphorylation, in which we combined phosphoprotein enrichment, by immobilized metal affinity chromatography (IMAC), with two-dimensional gel electrophoresis and mass spectrometry, in order to identify the phosphoproteins regulated in the striatum of PTN knockout, MK knockout and wild type mice treated with a single dose of cocaine (15 mg/Kg, i.p.). We identified 7 differentially expressed phosphoproteins: 5'(3')-deoxyribonucleotidase, endoplasmic reticulum resident protein 60 (ERP60), peroxiredoxin-6 (PRDX6), glutamate dehydrogenase 1 (GLUD1), aconitase and two subunits of hemoglobin. Most of these proteins are related to neurodegeneration processes and oxidative stress and their variations specially affect the PTN knockout mice, suggesting a protective role of endogenous PTN against cocaine-induced neural alterations. Further studies are needed to validate these proteins as possible targets against neural alterations induced by cocaine.

Keywords: Cocaine-induced neurotoxicity; midkine; oxidative stress; Parkinson's disease; pleiotrophin; phosphoproteomics.

1. INTRODUCTION

Pleiotrophin (PTN) and midkine (MK), two highly redundant in function cytokines (Herradon et al. 2005), are survival factors for dopaminergic neurons and capable to induce the differentiation of stem cells to dopaminergic neurons (Herradon and Ezquerra 2009; Muramatsu 2011). Pleiotrophin and MK are highly upregulated in different brain areas relevant to drug addiction such as hippocampus, striatum, cingulate cortex, fronto-parietal cortex and prefrontal cortex after administrations of different drugs like nicotine, amphetamine, cannabis, alcohol and morphine (Mailleux et al. 1994; Le Greves 2005; Ezquerra et al. 2007; Flatscher-Bader and Wilce 2006, 2008). Regarding psychostimulants, we have recently demonstrated that MK and PTN regulate drug-induced addictive behavior as seen in Conditioned Place Preference (CPP) experiments designed to test drug conditioning effects. In those experiments it was found that PTN genetically deficient (PTN-/-) mice maintain amphetamine-seeking behavior longer than wild type (WT+/+) and MK genetically deficient (MK -/-) mice (Gramage et al., 2010a; Martin et al., 2013); whereas, MK -/- mice show a significant delay in the extinction of cocaine-induced CPP (Gramage et al., 2013b). The influence of PTN and MK on the effects of psychostimulants has been also extended to these drugs-induced neurotoxicity. For instance, it has also been described that PTN prevents cocaine-induced cytotoxicity in NG108-15 and PC12 cell cultures (Gramage et al., 2008; Herradon et al., 2009). Moreover, amphetamine induces a significantly enhanced astrocytosis in the striatum of PTN-/- and MK -/-mice and also a loss of striatal dopaminergic terminals in PTN-/- mice compared to WT mice (Gramage et al. 2010a; 2010b; 2011).

Some of the proteins involved in the mechanism of action of PTN and MK have been suggested to be key for the neuroprotective roles of these cytokines against psychostimulants-induced neurotoxicity (Gramage and Herradon, 2011). One of the common receptors for PTN and MK is the Receptor Protein Tyrosine Phosphatase (RPTP) β/ζ (Meng et al., 2000; Sakaguchi et al., 2003). PTN and MK bind RPTPβ/ζ and inactivate the intrinsic tyrosine phosphatase activity of RPTPβ/ζ leading to a rapid increase in the steady state levels of tyrosine phosphorylation of substrates of RPTPβ/ζ (see review by Herradon and Ezquerra, 2009), many of which have been found to be very important for PTN and MK neuroprotective effects (Gramage and Herradon, 2011). In an effort to further dissect the different mechanisms of action triggered by PTN and MK to exert these protective roles against psychostimulant neurotoxicity we have recently compared the striatal phosphoproteome of WT+/+, PTN-/- and MK-/- after a neurotoxic treatment with amphetamine (Gramage et al., 2013b). In that study, we identified 13 differentially expressed phosphoproteins that are judged to be relevant in the neuroprotective roles of PTN and MK (Gramage et al., 2013b). Interestingly, 4 of these phosphoproteins, annexin A7, COP9 signalosome subunit 5, aldehyde dehydrogenase family 1 member A1 and creatine kinase U-type, were known to be involved in Parkinson's disease (PD), a result of significant importance since PTN and MK have also been shown to limit PD progress in animal models (Taravini et al., 2011; Prediger et al., 2011; Gombash et al., 2012) and have been suggested to be among the important genetic factors possibly preventing the development of PD in methamphetamine abusers (Gramage and Herradon, 2011; Callaghan et al., 2012).

Cocaine is another psychostimulant whose abuse has been connected to PD. Cocaine has been shown to increase the levels of α-synuclein, the main component of Lewy bodies in PD patients, in dopaminergic neurons (Ziolkowska et al., 2005; Qin et

al., 2005; Mash et al., 2003); and, recently, it has been confirmed the dose dependent toxicity of α-synuclein and its ability to induce neurodegeneration in dopaminergic neurons and other neuronal populations (Sánchez-Guajardo et al., 2013). In the present work we aimed to extend our knowledge on the molecular mechanisms triggered by PTN and MK to modulate psychostimulant neurotoxic and addictive effects by comparing the striatal phosphoproteome of WT+/+, PTN -/- and MK -/- mice, 24 hours after treatment with a single dose of cocaine (15 mg/kg, i.p.). We chose this treatment because acute administrations of cocaine (5 – 20 mg/Kg) are known to efficiently induce rewarding effects in rodents (Tzschentke, 2007) and the dose used in our studies has been proved to induce different addictive behaviors in the same mouse genotypes used in the present study (Gramage et al., 2013a). In addition, it is interesting to note that a single dose of cocaine within the same dose range has been shown to down-regulate mitochondrial genome and to increase the mitochondrial hydrogen peroxide generation together with a reduced functioning of the mitochondrial complex I in the striatum in response to cocaine (Dietrich et al., 2005). In a similar manner to studies recently performed by our group with amphetamine (Gramage et al., 2013b), we sought the identification of novel phosphoproteins differentially regulated in the striatum by cocaine administration depending on the presence of endogenous PTN or MK by employing a proteomic approach in which we combined immobilized metal affinity chromatography (IMAC) for phosphoprotein enrichment, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for protein separation and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry for protein identification.

2. METHODS AND EXPERIMENTAL PROCEDURES

2.1. PTN and MK genetically deficient mice

PTN-/- and MK-/- mice were kindly provided by Dr. Thomas F. Deuel (The Scripps Research Institute, La Jolla, CA). PTN-/- mice were generated as previously described (Amet et al. 2001; Del Olmo et al., 2009). The PTN gene consists of five exons encoding an 18-kDa protein with a 32 amino acid signal peptide. The replacement targeting vector generated a PTN null allele (PTN 2-4neo) by deleting exons 2 to 4. MK-/- mice were generated as previously described by using a basic vector to target a part of exon 1, intron 1 and a part of exon 2 of MK (Ezquerra et al., 2005, 2006; Nakamura et al., 1998). Male PTN-/-, MK-/- and WT+/+ mice on a C57BL/6J background were used at 8-10 weeks of age (20-25 g). Animals were carefully distributed so the average of the animal's age was similar in every experimental group. The genotypes of PTN-/- mice were confirmed by polymerase chain reaction using as primers 5'-GAT TGA ACA AGA TGG ATT GC-3' forward and 5'-CAT TTA GGC AAA CAG GAA GGA CG-3' reverse to generate a cDNA of ~0.7 kb detected in agarose gels from genomic DNA extracted from tails of PTN-/- and WT mice. The genotypes of the MK-/- mice were confirmed with the polymerase chain reaction using as primers 5'-ATC GGT TCC AAG TCC TCC CTC CGT C-3' forward and 5'-CAC CTT CCT CAG TTG ACA AAG ACA AGC-3' reverse to generate from genomic DNA extracted from tails of MK-/- and WT mice a cDNA of ~0.7 kb.

All the animals used in this study were maintained in accordance with European Union Laboratory Animal Care Rules (86/609/ECC directive) and the protocols were approved by the Animal Research Committee of USP-CEU.

2.2. Cocaine treatment

WT+/+, PTN-/- and MK-/- mice (n=8 per group) were administered with a single dose of 15 mg/kg, (i.p) of cocaine HCl (Alcaliber, Madrid, Spain) or saline (control, 10ml/kg). The dose of cocaine was chosen because it was proved to be effective in the induction of cocaine-seeking behavior in place conditioning studies performed in our laboratory with the same mouse genotypes (Gramage et al., 2013b). In addition, a similar acute dose (20 mg/kg) increases the production of reactive oxygen species in the striatum of rats (Dietrich et al., 2005) which is relevant in terms of cocaine-induced neurotoxic effects.

The six experimental groups depending on genotype and treatment were WT+/+ saline (WS), WT+/+ cocaine (WC), PTN-/- saline (PS), PTN-/- cocaine (PC), MK-/- saline (MS), and MK-/- cocaine (MC). 24 hours after administration of cocaine or saline, mice were sacrificed, their brains removed, the striatum dissected and preserved at -80°C.

2.3. Phosphoprotein identification: Proteomic analysis

Since we were interested in the identification of differentially phosphorylated proteins we used a proteomic approach, previously employed in our laboratory (Gramage et al., 2013a), in which we combined phosphoprotein enrichment, by immobilized metal affinity chromatography (IMAC), with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. This approach has been previously used by others for nearly identical experimental purposes (Lee et al., 2010; Talvas et al., 2008). Finally, some of the differentially phosphorylated

proteins detected by the proteomic techniques were tested in Western blots in individual samples.

2.3.1. Extraction and enrichment of phosphoproteins

For the extraction and enrichment of phosphoproteins we used the Pierce Phosphoprotein Enrichment Kit (Thermo Scientific, USA), which is based on a metal affinity chromatography (IMAC) and results in highly specific and efficient purification of phosphoprotein containing phosphotyrosine, phosphoserine and phosphothreonine residues (Nilsson et al. 2010). We followed the manufacturer's recommendations with slight modifications routinely used in our laboratory (Gramage et al., 2013a; Castillo et al, 2009). In brief, tissue samples (n=8) of each experimental group were pooled and homogenized by sonication (30 s bursts) with an ultrasonic probe (Dr. Hielscher, Germany), in 1mL of the Lysis/Binding/Wash Buffer with CHAPS (0.25%), provided by the kit, 10 μL of Halt Protease Inhibitor Single-Use Cocktail EDTA-Free (Thermo Scientific, USA), and 10 μL of Halt Phosphatase Inhibitor Cocktail (Thermo Scientific, USA). The samples were kept on ice to prevent over-heating during sonication. Then the samples were centrifuged (10,000 rpm, 20 min, 4°C) and the supernatants collected. Supernatants, containing up to 4 mg of total protein, were then applied to a column of the kit that contained a proprietary enrichment gel and buffer, for phosphoprotein enrichment. Samples were then incubated in the column for 30 min at 4°C and washed with the Lysis/Binding/Wash Buffer with CHAPS (0.25%) to remove unbound proteins. Bound proteins were eluted with five column washes of 1 mL of the elution buffer provided in the kit. The pooled elution fractions were placed into the concentrator columns of the kit and centrifuged (1000 rpm, 4°C) for 60 min or until the sample volume was 150 μL, approximately. The concentrated phosphoprotein-enriched samples were desalted by acetone (J.T. Baker, USA) precipitation (80% v/v, -20°C, overnight).

Precipitates were finally re-suspended to a final protein concentration of 0.27 μg/μL, with a buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 100 mM DTT, Bio-Lyte 3/10 ampholite 0.02% v/v, and a trace of bromophenol blue (Bio- Rad, USA). Protein concentration was measured by the Pierce BCA Protein Assay Kit (Thermo Scientific, USA).

2.3.2. 2D-PAGE

For protein separation, we employed the 2D gel electrophoresis protocol routinely used in our laboratory (Gramage et al., 2013a; Castillo et al., 2009). Briefly, 300 μl of each sample obtained in the previous step were taken for the rehydration and simultaneous loading of the proteins on an IPG strip (17 cm, 3–10 NL, Bio-Rad, USA) at 50 V, 20 °C, for 12 h in a PROTEAN IEF cell (Bio-Rad, USA). Then, the voltage was increased to 10000 V and focused for a total of 60000 Vh. Prior to SDS-PAGE, the strips were equilibrated in a solution containing 6 M urea, 2% SDS, 0.375 M Tris–HCl (pH 8.8), and 20% glycerol (Bio- Rad, USA). The equilibration consisted of two steps (10 min each), DTT (2%, Bio- Rad, USA) was added in the first step and iodoacetamide (2.5%, Bio- Rad, USA) in the second one to the equilibration solution. The SDS-PAGE was run in polyacrylamide gels (12%, 180 x 200 x 1 mm), at 200 V, 20 °C, for 6 h in a PROTEAN Plus Dodeca cell (Bio-Rad, USA). Then, the gels were stained with the ‘‘Silver Stain’’ kit (Bio-Rad, USA), according to manufacturer’s protocol, which is based on the method described by Sinha et al. (2001). Silver staining was performed, in a Dodeca Stainer (Bio-Rad, USA).

Gels (5 for each experimental group) were scanned using the densitometer GS-800 (Bio-Rad, USA). Spots were detected, quantified and matched automatically with the PDQuest v8 software from (Bio-Rad, USA), and manually checked. Normalization of the optical density of each spot and statistical analysis was conducted as described

before (Castillo et al., 2009). Spots that showed statistical differences in optical density ($P<0.05$) between two groups, and could be well-detected visually in the gels were cut out using pipette tips for mass spectrometry identification.

2.3.3. Mass spectrometry analysis of protein spots

Proteins selected for analysis were in-gel reduced, alkylated and digested with trypsin (Roche, Germany) according to Shevchenko et al. (1996). After digestion, the supernatant was collected and 1 µl was spotted onto a matrix-assisted laser desorption/ionization (MALDI) sample plate and allowed to air-dry at room temperature. Then, 0.4 µl of a 3 mg/ml solution of alpha-cyano-4 hydroxy-cinnamic acid in 50% acetonitrile, 0.1 % TFA (Sigma, Spain) were added to the dried peptide digest spots and allowed again to air-dry at room temperature. MALDI-time of flight (TOF) mass spectrometry analyses were performed at the Proteomics Facility UCM-PCM of Madrid, a member of ProteoRed network. A 4800 Plus Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, MDS Sciex, Canada) was used, operated in positive reflector mode with an accelerating voltage of 20,000 V. All mass spectra were calibrated internally using peptides from the auto digestion of trypsin. The analysis by MALDI-TOF/TOF mass spectrometry produces peptide mass fingerprints and the peptides observed with a Signal to Noise greater than 10 can be collated and represented as a list of monoisotopic molecular weights. Proteins ambiguously identified by peptide mass fingerprints, were subjected to tandem mass spectrometry sequencing analyses. So, from the mass spectrometry spectra suitable precursor were selected for tandem mass spectrometry analyses with CID on (atmospheric gas was used) 1 Kv ion reflector mode and precursor mass Windows +/- 5 Da. The plate model and default calibration were optimized for the tandem mass spectrometry spectra processing.

For protein identification, the non-redundant Uniprot/Swiss-Prot (release 57.15; 535698 sequences; 190107059 residues) database was searched using MASCOT 2.1 (matrixscience.com) through the Global Protein Server v3.6 from Applied Biosystems.

Search parameters were: carbamidomethyl cystein as fixed modification and oxidized methionine as variable modification; peptide mass tolerance 50ppm for peptide mass fingerprints searched and 80–100 ppm for tandem mass spectrometry searched; 1 missed trypsin cleavage site; tandem mass spectrometry fragments tolerance 0.3 Da

We accepted positive protein identification when the probability based Mowse Score was greater than the score fixed by MASCOT as significant with $p < 0.05$. We also checked that theoretical MW and pI values were similar to the experimental ones.

2.3.4. Western blot

The phosphoprotein enrichment by IMAC requires employing high amounts of proteins, since typical yields after IMAC are around 10 % of the total protein content. Therefore, we had to pool the striatum from different mice of the same experimental group for our proteomic studies. In addition, IMAC results in enrichment of phosphoproteins containing phosphotyrosine, phosphoserine and phosphothreonine residues (Nilsson et al. 2010) which could mask regulation of phosphorylation levels of specific residues, being of particular interest in this case phosphotyrosine residues which are highly regulated by the actions of PTN/MK on RPTPβ/ζ (Meng et al., 2000). Therefore, previously described (Gramage et al., 2013a), phosphotyrosine levels in target proteins found to be regulated in the Proteomics assays were tested in Western blots of striatum from individual samples of every experimental group.

After sacrifice, striatum from saline- and cocaine-treated PTN-/-, MK-/- and WT mice (n = 4/group), different from those used in Proteomics studies, were rapidly

dissected, frozen in dry ice and stored to -80ºC until the protein extraction procedure. Tissue samples were homogenized in RIPA buffer and protein extracted in presence of protease inhibitors and a phosphatase inhibitor cocktail (Sigma, Madrid, Spain). Total protein was quantified by the Bradford protein assay (Pierce, Rockford, IL). Equilibrated protein samples were mixed with loading buffer (60mM Tris pH 6.8, 10% glycerol, 5% SDS, 0.65% β-mercaptoethanol, and 0.01% bromophenol blue; Bio-Rad, USA), boiled for 5 minutes, and loaded onto 10% polyacrylamide gels as appropriate. The gels were transferred to nitrocellulose membranes that were blocked with 50mM Tris, 150mM NaCl, 0.1% Tween-20 (TBS-T, Bio-Rad, USA) and 5% non-fat milk for 30 min, and then probed with anti-phospho-Tyr (1:500) antibodies (Cell signaling, Danvers, MA). Membranes were then reprobed with anti-endoplasmic reticulum resident protein 60 (ERP60) (1:500) antibodies (Abcam, Cambridge, UK) or anti-aconitase (1:1,000) antibodies (Abcam, Cambridge, UK) to confirm the identity of the protein and to normalize the levels of tyrosine phosphorylation by the content of the target proteins in each of the individual samples. In order to normalize total protein levels of each of these proteins, membranes were re-probed with anti-actin antibodies at a 1:2,000 dilution (Chemicon, Temecula, CA). After 3 washes in TBS-T, the membranes were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase diluted 1:5,000 in TBS-T with 5% non-fat milk for 30 min. The membranes were washed 3 times in TBS-T and the immunoreactive proteins were visualized using the ECL Enhanced method according to the manufacturer's instructions (Amersham, San Francisco, CA). Phospho-Tyr levels were quantified by densitometry in each animal sample using Image Lab image acquisition and analysis software (Bio-Rad, Hercules, CA) and normalized with total ERP60 and aconitase protein levels. Data are presented as mean ± standard error of the mean (S.E.M.) and

were analyzed using two-way ANOVA. Relevant differences were analyzed pair-wise by post-hoc comparisons with Bonferroni's post-hoc tests, considering genotype and treatment as variables. $P < 0.05$ was considered as statistically significant. All statistical analyses were performed using graphpad prism 4 program (San Diego, CA, USA).

3. RESULTS

For the identification of differentially phosphorylated proteins in WT+/+, PTN-/- and MK-/- mice treated with cocaine (15 mg/kg, i.p.), the samples obtained after pooling 8 striatum of every experimental group (WS, WC, PS, PC, MS and MC) were first enriched in phosphoproteins by IMAC. The phosphoprotein yield ranged from 8.0 to 12.0 % of the total protein content which is in agreement with previous studies (Gramage et al., 2013a). Proteins were then separated by 2D-PAGE. The gels (n = 3-5 per group) obtained from the 6 experimental groups were analyzed simultaneously and matched in the same set. The silver staining allowed the visualization of faint spots in the gels, however for the statistical analysis we considered only well-resolved spots, with an optical density higher than 50 a.u. that could be well visualized, manually excised and, therefore, analyzed by mass spectrometry. Of all spots that showed significant differences in normalized optical density among the different experimental groups, we selected those with an expression fold-change of 1.5 or more. Finally, 7 spots were successfully identified by either peptide mass fingerprinting or tandem mass spectrometry (Table 1). These spots are indicated in one representative gel showed in Figure 1. The phosphorylation in different residues of all the proteins included in the identified spots has been previously demonstrated according to PhosphositePlus (Hornbeck et al., 2012; see Table 1).

The comparison of the 2-D patterns between different treatments within the same genotype resulted in 4 proteins showing significant differences in optical density (Table 2). Thus, hemoglobin subunit beta-2 (HBB2) was significantly upregulated in WT +/+ and MK -/- after the treatment with cocaine, the same variation was observed in aconitase in WT +/+ mice. In contrast, cocaine induced a significant dowregulation in the expression of ERP60 and peroxiredoxin-6 (PRDX6) in PTN -/- mice.

When comparing genotypic differences within the same treatment (saline or cocaine), we identified significant differences in the optical density of 7 proteins (Table 2). PRDX6 and hemoglobin subunit beta-1 (HBB1) were detected in the comparison of WS *vs* PS and MS groups, respectively. HBB2 was significantly upregulated in MC compared with WC group. When compared with WC, PC group showed significant differences in the expression of 5'(3')-deoxyribonucleotidase (NT5C), ERP60, glutamate dehydrogenase 1 (DHE3), HBB1, HBB2 and aconitase. Finally NT5C and PRDX6 were significantly downregulated in MS compared with PS group, and, in contrast, ERP60, PRDX6 and HBB2 were significantly upregulated in MC *vs* PC group.

As representative of these proteins, we chose to further study the pattern of tyrosine phosphorylation of ERP60 and aconitase, by performing Western blots in samples from 4 individual animals per experimental group. We chose ERP60 because another member of its family, endoplasmic reticulum resident protein 29 (ERP29), was detected as significantly modified in a similar phosphoproteomic analysis of the striatum of the same three genotypes of animals treated with amphetamine (Gramage et al., 2013a). Aconitase was chosen because, as described in the discussion section, this protein is involved in neurodegenerative diseases, including PD. Neither the non-phosphorylated nor the tyrosine-phosphorylated form of aconitase showed significant changes of expression between the different experimental groups (Figure 2). However, in the case of ERP60, Western blots showed a similar pattern of expression to that uncovered in the proteomic analysis. Thus the two-way ANOVA analysis revealed a significant interaction of genotype and treatment on the tyrosine phosphorylation levels normalized by total amounts of ERP60 ($F(2,22)=4.237$; $P=0.0278$) (Figure 3). Interestingly, and identically to the data obtained in the proteomic studies, post hoc comparison showed

that PTN -/- was the only genotype in which cocaine induced a significant decrease of the tyrosine phosphorylated form of ERP60. In addition, the two-way ANOVA analysis uncovered a significant effect of the genotype on total ERP60 protein levels normalized by actin levels ($F(2,13)=7.408$; $P=0.0071$) (Figure 3).

4. DISCUSSION

We have recently proved that neurotoxicity induced by amphetamine in the striatum of PTN-/- and MK-/- mice is accompanied by specific alterations in the phosphoproteome of these animals compared to WT+/+ mice (Gramage et al., 2013b). In the present work we have extended the study of psychostimulant-induced alterations in the striatal phosphoproteome of these mice by analyzing the effects induced by cocaine. We have successfully identified 7 phosphoproteins significantly affected by cocaine treatment, genotype or both. Interestingly, despite the well described functional redundancy between PTN and MK (Herradon et al., 2005), most of the proteins identified in this study were differentially affected in both genotypes which could be relevant to uncover the molecular mechanisms underlying the previously described functional differences between PTN and MK in the modulation of the addictive and neurotoxic effects induced by psychostimulants like amphetamine and cocaine (Gramage et al., 2010a; 2010b; 2011, 2013b; Martin et al., 2013).

Some of the concerns with the methodology used in our study are the lack of discrimination between the different phosphorylated residues obtained by the phosphoprotein enrichment by IMAC and that the possibility of detection of non-phosphorylated proteins cannot be ruled out. Therefore, following protocols previously reported (Gramage et al., 2013b), we measured the levels of total and tyrosine phosphorylated forms of two of the identified proteins (ERP60 and aconitase) by Western blots in the striatum from individual samples of every experimental group. ERP60 was chosen because we previously described a modification in the expression of ERP29 in the phosphoproteomic study comparing the striatum of WT+/+, PTN-/- and MK-/- mice treated with amphetamine (Gramage et al., 2013b); moreover, in that study we found a similar pattern of expression of the tyrosine phosphorylated form of ERP29

when we compared the proteomic and the Western blot results. In the present study, we found again a similar pattern of ERP60 expression in Western blot to that observed in the proteomic analysis confirming that cocaine induces a significant decrease of the tyrosine phosphorylated form of ERP60 only in PTN-/- mice. Additionally we detected a significant increase of the total form of ERP60 in PTN-/- and MK-/- mice when compared to WT+/+ mice.

The ERP60 protein has many synonyms, including ERP57, glucose-regulated protein 58-kD and protein disulfide-isomerase A3 and is a stress-responsive molecular chaperone located mainly in the endoplasmic reticulum. However, ERP60 is present in many other subcellular locations, where it is involved in a variety of functions (Turano et al., 2011). ERP60 has been linked to numerous diseases, including neurodegenerative diseases, such as Alzheimer' disease and PD (Stemmer et al., 2013; Tohda et al., 2012; Shi et al., 2011; Hurley et al., 2013; Verhaar et al., 2012; Lessner et al, 2010), where it could have a neuroprotective role, as demonstrated against prion neurotoxicity (Hetz et al., 2005). Moreover, recently, Pendyala et al. (2012) have reported a significant upregulation of ERP60 in the caudate and hippocampus of monkeys as well as in the striatum of mice after treatment with methamphetamine and have demonstrated that ERP60 protects against methamphetamine-induced cell toxicity and methamphetamine-induced intracellular reactive oxygen species production in a neuroblastoma cell line. These data led to the hypothesis that ERP60 plays an important cellular neuroprotective role against a toxic drug. Accordingly, the reduction of the phosphorylated form of ERP60 observed in the PTN -/- mice treated with cocaine would make these animals more susceptible to the putative damage produced by the drug. However, additional studies on the functional consequences of tyrosine phosphorylation of ERP60 are

needed to clarify the contribution of PTN on the putative neuroprotective role of ERP60 against cocaine-induced neurotoxicity.

We also measured the expression levels of aconitase by Western blot. Aconitase is located mainly in the mitochondria where it functions as key enzyme in the tricarboxylic acid cycle in the presence of iron (Gille and Reichmann 2011). Aconitase is highly sensitive to oxidative inactivation by superoxide radicals that are produced in the mitochondria because of its labile iron atom (Gardner and Fridovich, 1991), thus a decrease in brain aconitase activity is considered a marker of oxidative protein damage (Liang et al., 2000). In addition, a decrease in aconitase activity has been observed in several neurodegenerative diseases associated with the development of oxidative stress, including Huntington's disease, Friedreich ataxia, PD (Schapira, 1999; Gille and Reichmann, 2011; Ghosh et al., 2010), and Alzheimer's disease (Raukas et al., 2012). Our Western blot data do not show any significant changes between genotypes or treatments neither in the levels of tyrosine phosphorylation of aconitase nor in the total protein levels, suggesting the differences identified in our proteomic study could be caused by differences in the phosphorylation levels of other residues (i.e. serine or threonine). However, it has to be noted that decrease of aconitase activity in neurodegenerative disorders is consistent with the significant reduction of the phosphorylated enzyme observed in our proteomic study in cocaine-treated PTN-/- mice when compared with WT+/+ mice, which could reflect again a higher susceptibility of PTN-/- mice against psychostimulant-induced neurotoxicity (Gramage and Herradon, 2011).

In our proteomic study we also detected a significant decrease in the phosphorylated form of PRDX6 in PTN-/- mice treated with cocaine when compared to those treated with saline. Interestingly, in previous studies we have showed that the

levels of PRDX6 and other peroxiredoxins are differentially regulated in rodents according to the rewarding effects induced by cocaine (Del Castillo et al., 2009; Gramage et al., 2013a). PRDX6 belongs to the peroxiredoxin family of sulfhydryl-dependent peroxidases that have well-established peroxide-scavenging activity and neuroprotective effects in different neurological disorders (Zhu et al., 2012). Thus, for instance, altered expression of PRDX6 has been detected in the brain of schizophrenia, PD, and Alzheimer's disease patients (Martins-de-Souza et al., 2009; Krapfenbauer et al., 2003; Power et al., 2008); however, recently, it has been suggested that the overexpression of PRDX6 could accelerate the development of Alzheimer's disease (Yun et al., 2013). On the other hand, accumulating evidence indicates that low levels of expression of PRDX6 contribute to pathophysiology of cells and tissues, and this involves an increase in reactive oxygen species levels. For instance PRDX6 attenuates TNFα-, glutamate- and hypoxia-induced retinal ganglion cells death, by limiting reactive oxygen species and maintaining Ca^{2+} homeostasis (Fatma et al., 2008; Tulsawani, et al., 2010). Taken this into account, it would be plausible to think that in our study, the reduction of PRDX6 observed in the PTN-/- mice after the treatment with cocaine would contribute to the higher vulnerability of mice lacking endogenous PTN to drugs of abuse-induced neurotoxic effects (Herradon et al., 2009; Gramage and Herradon, 2011). Nevertheless, in this case is crucial to know the influence of tyrosine phosphorylation on the activity of the protein since PRDX6 is a bifunctional protein with both peroxidase and phospholipase A2 activities. The peroxidase activity of PRDX6 is unaffected by phosphorylation (Wu et al., 2009); however, the phosphorylation of threonine in position 177 results in an activation of the phospholipase A_2 activity of PRDX6 (Rahaman et al., 2012) and it has been described that phospholipases A_2 increase during neurodegeneration (Farooqui et al., 2004).

Our phosphoproteomic study also revealed high levels of glutamate dehydrogenase 1 (GLUD1) in the PTN-/- mice, being these levels significantly higher in the PTN -/- mice treated with cocaine compared to WT+/+ mice. GLUD1 appears to be a key mitochondrial enzyme in regulating the amount of glutamate synthesized and released from neurons; thus overexpression of GLUD1 in neurons led to increases in the levels of glutamate in several brain areas, including striatum (Bao et al., 2009). Excessive excitation of neurons by glutamate causes neurotoxicity, a process linked to several neurodegenerative diseases (Rahn et al., 2012). Indeed, Glud1 gene has been identified as a memory-related gene in the hippocampus (Cavallaro et al., 1997) and dysregulation of a related Glud gene expressed in the human brain, Glud2, has been associated with early onset of PD (Plaitakis et al., 2010). The molecular mechanisms of neurodegeneration caused by exposure to glutamate excess include the production of reactive oxygen species (Aarts et al., 2003). Taken into account that it has been described that acute cocaine increases the production of reactive oxygen species in the striatum (Dietrich et al., 2005), this observation contributes to support the alterations in proteins related to oxidative stress detected in our study (i.e. aconitase, ERP60, PRDX6 and GLUD1), especially in the PTN-/- mice treated with cocaine.

We also observed a significant increase in the phosphorylated form of 5'(3')-deoxyribonucleotidase, cytosolic type (NT5C) in PTN-/- mice treated with cocaine compared to WT+/+ mice. NT5C was also significantly increased in PS compared to MS mice. NT5C belongs to the 5′-nucleotidases family that dephosphorylates non-cyclic nucleoside monophosphates to nucleoside and inorganic phosphate (Bianchi and Spychala, 2003). The increase in the expression of the phosphorylated form of NT5C observed in PTN -/- mice is consistent with the idea that these animals are more susceptible to cocaine-induced damage, since 5′-nucleotidase hyperactivity has been

associated with neurological and developmental disorders (Pesi et al., 2005; 2008). This idea is also in agreement with our previous finding that PTN prevents cocaine-induced cytotoxicity in NG108-15 and PC12 cell (Gramage et al., 2008).

Finally, we also detected variations in the expression of the phosphorylated form of two subunits of hemoglobin (HBB1 and HBB2). It has been suggested that phosphorylation of hemoglobin might be a general mechanism for regulating oxygen exchange (Hornbeck et al., 2008); however we do not think that the small changes detected in our study, although significant, could have any physiological or pathological consequences taken into account the high level of expression of these proteins.

5. CONCLUSIONS

The present study identify for the first time very specific phosphoproteins related to neurodegeneration processes that are differentially regulated in the mouse striatum by cocaine administration depending on the endogenous expression of PTN/MK. Most of these proteins are related to oxidative stress and their variations specially affect to PTN-/- mice, which could suggest a protective role of endogenous PTN to the cocaine-induced neural alterations which correlates with the known neuroprotective effects of PTN against cocaine-induced cytotoxicity in cultures. However, further studies, including the influence of phosphorylation, are needed to validate these proteins as possible targets against neural alterations induced by cocaine.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Representative silver stained 2D gel of the phosphoproteome from the mouse striatum. Spots labelled with numbers showed significant differences in normalized optical density after comparing the 6 groups of animals and were identified by mass spectrometry.

Figure 2. Levels of phosphorylation in tyrosine of aconitase in the striatum of PTN-/-, MK-/- and WT+/+ mice after cocaine administration. The levels of phosphorylation of Tyr in saline (Sal) and cocaine-treated PTN-/-, MK-/- and WT+/+ mice (n = 4/group) were determined in Western blots probed with anti-phospho-Tyr antibodies. Total aconitase and Actin amounts were determined using anti-aconitase and anti-actin antibodies. Upper graph: Ratio P-Tyr/ERP29 of optical density (OD) measurements corresponding to the phosphorylated form and total aconitase protein levels respectively. Lower graph: Ratio aconitase/Actin of optical density (OD) measurements corresponding to the total aconitase and actin protein levels respectively. Data show mean ± S.E.M of the four individual samples from every experimental group.

Figure 3. Levels of phosphorylation in tyrosine of endoplasmic reticulum resident protein 60 (ERP60) in the striatum of PTN-/-, MK-/- and WT+/+ mice after cocaine administration. The levels of phosphorylation of Tyr in saline (Sal) and cocaine treated PTN-/-, MK-/- and WT mice (n = 4/group) were determined in Western blots probed with anti-phospho-Tyr antibodies. Total ERP60 and Actin amounts were determined using anti-ERP60 and anti-actin antibodies. Upper graph: Ratio P-Tyr/ERP60 of optical density (OD) measurements corresponding to the phosphorylated form and total ERP60 protein levels respectively. Lower graph: Ratio ERP60/Actin of optical density (OD) measurements corresponding to the total ERP60 and actin protein levels respectively. Data show mean ± S.E.M of the four individual samples from every experimental group.* $P<0.05$ vs saline.