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Differential phosphoproteome of the striatum from pleiotrophin knockout and midkine knockout mice treated with amphetamine: Correlations with amphetamine-induced neurotoxicity

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ABSTRACT

The neurotrophic factors pleiotrophin (PTN) and midkine (MK) have been shown to modulate amphetamine-induced neurotoxicity. Accordingly, PTN−/− and MK−/− mice show an increased vulnerability to amphetamine-induced neurotoxic effects. In an effort to uncover new pharmacological targets to prevent amphetamine neurotoxic effects, 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−/−, MK−/− and wild type (WT) mice treated with amphetamine. We identified 13 differentially expressed phosphoproteins that are judged to be relevant in the neuroprotective roles of PTN and MK against amphetamine-induced neurotoxicity. It is very interesting to note that 4 of these phosphoproteins, annexin A7 (ANXA7), COP9 signalosome subunit 5 (COPS5), aldehyde dehydrogenase family 1 member A1 (ALDH1A1) and creatine kinase U-type (CKMT1), are known to be involved in Parkinson's disease, a result of significant importance since PTN and MK have been also demonstrated to limit Parkinson's disease (PD) progress and have been suggested to be among the important genetic factors possibly preventing the development of PD in methamphetamine abusers. The data identify phosphoproteins differentially regulated by amphetamine treatment and/or the presence of endogenous PTN/MK which may be relevant mediators of PTN/MK neuroprotective effects against amphetamine-induced neurotoxicity. The data support further studies to validate the phosphoproteins here identified as possible new pharmacological targets to prevent amphetamine neurotoxic effects.

1. Introduction

Amphetamine and its derivatives are drugs that are globally abused and lead to addictive behavior and severe neurological damage (Callaghan et al., 2012; Yamamoto and Bankson, 2005). Hallmarks of neurotoxicity induced by these drugs include proliferation of astrocytes in striatum (Krasnova et al., 2005; Pu and Vorhees, 1995), apoptosis of striatal neurons and destruction of striatal dopaminergic terminals (Ares-Santos et al., 2012; Granado et al., 2008a, 2008b, 2010, 2011; Krasnova et al., 2005). Increasing efforts have led to the identification of different factors that efficiently modulate the neurotoxic effects of these drugs in an effort to uncover new pharmacological targets to prevent drug-induced neurotoxicity (Ares-Santos et al., 2012; Gramage et al., 2010a, 2010b, 2011; Granado et al., 2011; Herradon et al., 2009). Among them, the neurotrophic factors pleiotrophin (PTN) and midkine (MK) have been shown to limit amphetamine neurotoxic effects (Gramage and Herradon, 2011). Despite the significant functional redundancy of PTN and MK (Herradon et al., 2005), amphetamine treatment in mice genetically deficient in PTN (PTN−/−) resulted in

Abbreviations: ALDH1A1, aldehyde dehydrogenase family 1 member A1; ANXA7, annexin A7; ATP5A1, ATP synthase subunit alpha; ATP5D, ATP synthase subunit d; COPS5, COP9 signalosome subunit 5; CKMT1, creatine kinase U-type; ALAD, delta-aminolevulinic acid dehydratase; ERP29, endoplasmic reticulum resident protein 29; ERK, extracellular signal-regulated kinase; FAH, fumarylacetoacetase; ENO2, gamma-enolase; RAN, GTP-binding nuclear protein Ran; IMAC, immobilized metal affinity chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MK, midkine; MA, MK−/− amphetamine; MS, MK−/− saline; PD, Parkinson's disease; PGAM1, phosphoglycerate mutase 1; PTN, pleiotrophin; PS, PTN−/− saline; PA, PTN−/− amphetamine; RPTP, receptor protein tyrosine phosphatase; ALDH1A1, retinal dehydrogenase or aldehyde dehydrogenase family 1 member A1; CCT2, T-complex protein 1 subunit beta; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; WT, wild type; WA, WT amphetamine; WS, WT saline.

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increased astrocytosis in striatum and substantia nigra, increased loss of striatal dopaminergic terminals and, surprisingly, loss of tyrosine hydroxylase-positive neurons in the substantia nigra, an effect that is not observed in wild type (WT) mice (Gramage et al., 2010a, 2010b). On the other hand, amphetamine-induced striatal astrocytosis is enhanced in MK−/− mice whereas loss of dopaminergic terminals in the striatum was found to be similar to that observed in WT mice (Gramage et al., 2011). These different responses to amphetamine treatment in PTN−/−, MK−/− and WT mice suggest this as an interesting model to identify druggable downstream targets in the PTN/MK signaling pathways that modulate amphetamine neurotoxic effects.

One receptor that initiates both PTN and MK signaling pathways is the trans-membrane receptor protein tyrosine phosphatase (RPTP) β/ζ (Meng et al., 2000; Sakaguchi et al., 2003). The interaction of PTN/MK with RPTPβ/ζ inactivates 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). Fyn kinase, one of the substrates of RPTPβ/ζ whose levels of tyrosine phosphorylation are increased by MK/PTN, has been related to survival of dopaminergic neurons (Pariser et al., 2005a). Fyn has been shown to activate extracellular signal-regulated kinase (ERK) 1/2 signaling pathway by increasing the phosphorylation levels of ERK1/2 (Lovatt et al., 2006). Interestingly, we found a significant decrease in the phosphorylation levels of ERK1/2 in the striatum of amphetamine-treated PTN−/− compared to MK−/− and WT mice (Gramage et al., 2010a, 2010b, 2011), suggesting diminished striatal activation of ERK1/2 could underlie the increased amphetamine-induced striatal TH loss specifically found in PTN−/− mice. Thus, the data suggest that PTN and MK neuroprotective effects could be exerted by different molecular mechanisms.

Proteomics employs high-throughput technologies that allow the investigation of proteins on a large scale in terms of their expression, function, interactions or post-translational modifications (Li and Wang, 2007). In contrast to other techniques routinely used in our laboratory, such as Western blot or immunohistochemistry, the proteomic approach does not require preselected target proteins; consequently, the results should be more objective, and could lead to the identification of novel proteins involved in the effect studied (Mann, 2008). Given the mechanism of action triggered by PTN and MK to modulate the phosphorylation levels of different proteins involved in their downstream signaling pathways, we have now used a well-established proteomic approach to study protein phosphorylation (Lee et al., 2010; Talvas et al., 2008), which includes 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. In the present work, we have used this approach to perform a large scale study of protein phosphorylation in the striatum of amphetamine-treated PTN−/−, MK−/− and WT mice in an effort to dissect the different mechanisms of action triggered by PTN and MK to exert their neuroprotective roles against amphetamine-induced neurotoxicity in striatum.

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 2–4. 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 129/Ola × C57BL/6 J 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. Amphetamine treatment

WT, PTN−/− and MK−/− mice ($n = 8$ per group) were administered with four injections of amphetamine (Sigma, Spain) (10 mg/kg) or saline (control, 10 mL/kg) with a 2-h interval between injections. This dose regimen has been previously shown to produce differential neurotoxic effects in the striatum of PTN−/−, MK−/− and WT mice (Gramage et al., 2010a, 2010b, 2011).

The six experimental groups depending on genotype and treatment were WT saline (WS), WT amphetamine (WA), PTN−/− saline (PS), PTN−/− amphetamine (PA), MK−/− saline (MS), and MK−/− amphetamine (MA). Four days after administration of the first dose of amphetamine or saline, mice were sacrificed, since it has been previously reported that amphetamine-induced neurotoxicity in the striatum peaks 4 days after the drug administrations (Krasnova et al., 2005). Immediately after sacrifice, the mice brains were removed, the striatum dissected and preserved at −80 °C.

2.3. Phosphoprotein identification: proteomic analysis

To make possible the identification of differentially phosphorylated proteins we used a proteomic approach 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 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 (Castillo et al., 2009; Sorzano et al., 2008). 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 1 mL 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

2D gel electrophoresis was carried out as described previously (Castillo et al., 2009; Zhan and Desiderio, 2003a). 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 10,000 V and focused for a total of 60,000 Vh. Prior to SDS-PAGE, the strips were equilibrated in a solution