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Western blot detection of brain phosphoproteins after performing Laser Microdissection and Pressure Catapulting (LMPC)

Marlène Maitre[a,b], Valérie Roullot-Lacarrière[a,b], Pier Vincenzo Piazza[a,b], Jean-Michel Revest[a,b,*]

[a] INSERM U862, Neurocenter Magendie, Pathophysiology of Addiction group, Bordeaux F33077, France
[b] University of Bordeaux, Bordeaux F33077, France

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ABSTRACT

The Central Nervous System (CNS) is constituted of complex and specific anatomical regions that cluster together and interact with each other with the ultimate objective of receiving and delivering information. This information is characterized by selective biochemical changes that happen within specific brain sub-regions. Most of these changes involve a dynamic balance between kinase and phosphatase activities. The fine-tuning of this kinase/phosphatase balance is thus critical for neuronal adaptation, transition to long-term responses and higher brain functions including specific behaviors. Data emerging from several biological systems may suggest that disruption of this dynamic cell signaling balance within specific brain sub-regions leads to behavioral impairments. Therefore, accurate and powerful techniques are required to study global changes in protein expression levels and protein activities in specific groups of cells. Laser-based systems for tissue microdissection represent a method of choice enabling more accurate proteomic profiling. The goal of this study was to develop a methodological approach using Laser Microdissection and Pressure Catapulting (LMPC) technology combined with an immunoblotting technique in order to specifically detect the expression of phosphoproteins in particular small brain areas.

1. Introduction

The anatomical complexity of the Central Nervous System (CNS) allows highly specific and selective cellular responses of the brain. However, since these changes in cellular activities are located in small brain areas, their characterization is extremely difficult. Dissecting these sub-regions of interest thus bypassing the problem of the functional heterogeneity of the cerebral tissue is therefore a challenge. Indeed, cerebral structures are often composed of sub-regions with specific functional properties. For example, the hippocampal formation divided into distinct subfields referred to as CA1, CA2, CA3 and DG (Dentate Gyrus) plays a prominent role in some of the hippocampus-mediated behaviors (Abrous et al., 2005; Martin and Clark, 2007). Another example is the nucleus accumbens (NAc) which can be divided into two structures referred to as the Core and the Shell. These structures also have different morphologies and functions (Marinelli and Piazza, 2002; Di Chiara, 2002; Ikemoto, 2007).

Consequently, molecular mechanisms of protein-mediated physiologies/physiopathologies can only be understood once the specific cellular targets of these proteins have been identified. In this context, laser-assisted tissue microdissection is a method of choice which is much more precise than conventional manual dissection (Emmert-Buck et al., 1996). Two main laser-based microscope-aided systems of tissue microdissection have been developed namely laser capture microdissection and laser cutting microdissection. They are now marketed respectively by Life Technologies/Applied Biosystems, initially Arcturus a company formerly based in the USA (website: http://www.appliedbiosystems.com) and European-based companies such as Zeiss (website: http://www.zeiss.de/microdissection), Leica (website: http://www.leica-microsystems.com) or Molecular Machines & Industries (website: http://www.molecular-machines.com/products/lasermicrodissection.html), for review see Murray (2007) and Burgemeister (2005).

Thus, laser-based methods of microdissection coupled with real time quantitative PCR (qPCR) analysis has already been successfully applied to study messenger RNA (mRNA) expression from different tissue (Fend et al., 1999; Vincent et al., 2002; Jacquet et al., 2005; Bernard et al., 2009; Ou et al., 2010).

However, changes in the molecular activities of a cell or group of cells are not limited to changes in the quantities of mRNA but also involve changes in protein levels. Finally, a third level of regulation involves posttranslational modifications of proteins allowing the functional diversification of the proteome (i.e., phosphorylation, glycosylation, methylation, acetylation, etc.) (Han and Martinage,

* Corresponding author at: Institut de Physiopathologie de la Plasticité Neuronale, Inserm U862, Institut François Magendie, 146 rue Léo Saignat 33077 Bordeaux Cedex, France. Tel.: +33 5 57 57 36 72; fax: +33 5 57 57 36 69.
E-mail address: jean-michel.revest@inserm.fr (J.-M. Revest).

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1992; Walsh et al., 2005). Protein phosphorylation is mediated by the largest class of posttranslational modifying enzymes named protein kinases. This superfamily of proteins, also known as the kinome, is involved in one of the most popular cell signaling transduction mechanisms controlling complex processes both in prokaryotic and eukaryotic cells. Indeed, numerous data from the literature show that neuronal activity involves the modulation of kinase activities and that the disruption of the dynamic cell signaling balance between kinase and phosphatase activities leads to behavioral impairments (Greengard et al., 1993; Jovanovic et al., 1996; Atkins et al., 1998; Revest et al., 2005, 2010a,b; Barik et al., 2010). Consequently, accurate and powerful techniques such as laser-based systems of tissue microdissection are required to specifically isolate the cellular targets where these molecular events take place.

In this study, we used the well-adapted Western blot technique to study the phosphorylation of proteins. Detection of protein phosphorylation first requires dissecting sufficient quantities of material for further proteomic analysis and then developing an experimental protocol that preserves the functional integrity of the activated cells. Here, we describe a sensitive Western blot protocol coupled with the LMPC process guided by histological staining of the tissue sections that both preserves phosphorylation and allows the detection of phosphorylated proteins within small areas of the brain.

2. Materials and methods

2.1. Cell culture

The PC12 cell line (ATCC CRL-1721) derived from a transplantable rat pheochromocytoma was used. The PC12 cells were cultured in fresh, antibiotic-free medium (10% Foetal Bovine Serum) then trypsinized and counted up to the appropriate concentration (50×10^3, 10×10^3, 5×10^3, 1×10^3 cells/lane of gels) using a Malassez chamber.

2.2. Brain tissue samples

For all the experiments, 5–6 week-old Sprague–Dawley male rats (Charles River, Arbresle, France) were given an overdose of pentobarbital (0.1 ml/kg, Bayer, Germany). Brains were quickly dissected and snap-frozen in isopentane (#277258, Sigma–Aldrich, USA) at −40 °C and stored at −80 °C until use. All the experiments were conducted in strict compliance with the European Community Council Directive of 24 November 1986 (86/609/EEC), and approved by the Aquitaine-Poitou Charentes ethical committee.

2.3. Laser Microdissection and Pressure Catapulting (LMPC)

Ten micrometer thick coronal sections were carried out on fresh frozen tissue at −20 °C to prevent protein degradation using a CM3050 S microtome (Leica, Germany). Tissue sections were mounted on PEN-membrane 1 mm glass slides (#415101-4401-600, P.A.L.M. Microlaser Technologies AG, Zeiss, Germany). Frozen sections were fixed by incubation for 1 min in pre-cooled (−20 °C) 80% ethanol (EtOH, #818760, Merck, USA), then stained with either Harris's (#HHS32, Sigma–Aldrich, USA), Mayer's (#MHS32, Sigma–Aldrich, USA) hematoxylins or 1% cresyl violet stain (#C5042, Sigma–Aldrich, USA). Protease and phosphatase inhibitors were added in staining and in water solutions in order to minimize protein and posttranslational modification degradations during the process. Proteins were extracted from EtOH-fixed tissue since it yields excellent histomorphology and good preservation of macromolecules for further proteomic analysis (Ahram et al., 2003). Subsequently, sections were rinsed with water for 30 s, dehydrated in a series of pre-cooled EtOH baths: 50% EtOH (30 s), 70% EtOH (30 s) and 100% EtOH (1 min) then air-dried. Immediately after dehydration, laser assisted-microdissection was performed using a P.A.L.M. MicroBeam microdissection system version 4.0-1206 equipped with a P.A.L.M. RoboSoftware (P.A.L.M. Microlaser Technologies AG, Zeiss, Germany). We used an FTSS (Frequency Tripled Solid State) laser with a wavelength of 355 nm (UV) and a pulse of energy at 100 μJ. The two major features of ultraviolet LMPC is that the UV laser combines high photon density (cold laser) and low wavelengths, thus the heat generated during microdissection and therefore the impact on cellular biomolecules (DNA, RNA, and proteins) is minimal (Vogel et al., 2007). After laser microdissection along the circumscribed area, a defined laser pulse catapults the selected specimen out of the object plane into the cap of a collection device located above the tissue section. As this process works entirely without mechanical contact, it enables pure sample retrieval of morphologically defined origin with no cross-contamination (Vogel et al., 2007). The presence of captured tissue samples in the cap was checked under low magnification, then the samples were immediately stored at −80 °C until use. By repeating this procedure several times, the captured laser microdissected material could be accumulated in a single cap. Laser power, pulse frequency (1–100 Hz) and duration were adjusted to optimize capture efficiency. Microdissection was performed at 5× or 20× magnification. LMPC was performed for no longer than 30 min per slide.

Cell count analysis before laser-assisted microdissection was performed on a PC computer running Windows using the ImageJ program with specific plugins. ImageJ is a Java image processing program similar to the public domain NIH Image program developed at the U.S. National Institutes of Health and freely available on the Internet at http://rsb.info.nih.gov/ij/.

2.4. Protein extraction from PC12 and brain tissues

PC12 cells and LMPC brain samples were either directly homogenized in NuPAGE LDS Sample Buffer supplemented (#NP0007, Invitrogen, USA) with NuPAGE Reducing Agent (#NP 0004, Invitrogen, USA) or in RIPA buffer. RIPA buffer 1% Igepal CA-630 (#I-3021, Sigma, USA), 0.5% sodium deoxycholate (#D6750-100G, Sigma, USA), 0.1% SDS (Sodium Dodecyl Sulfate, #EU0660B, Euromedex, France) in PBS (Phosphate-buffered saline, #ET 330, Euromedex, France) was supplemented with 0.5 μl/ml of 1 M DTT (Dithiothreitol, #EU 0006-C, Euromedex, France), 5 μl/ml of both Protease Inhibitor Cocktail (PIC, #P8340) and phosphatase inhibitors (#P0044), both supplied by Sigma (USA). Protein concentrations were determined using the Pierce BCA Protein Assay kit (#23225, Pierce, USA). Microdissections of the various brain regions expressed in μm^3: 26×10^6, 5.2×10^6, 2.6×10^6 and 0.5×10^6, respectively contain similar protein concentrations and allowed loading by wells 15 μg, 3 μg, 1.5 μg and 0.3 μg of proteins. PC12 cells were suspended in 15 μl of NuPAGE LDS Sample Buffer supplemented with NuPAGE Reducing Agent. LMPC microdissected cells were lysed by adding 15 μl of NuPAGE LDS Sample buffer supplemented with NuPAGE Reducing Agent on top of the LMPC caps. Cell lysates with NuPAGE LDS Sample Buffer were then heat-denatured for 10 min at 70 °C and subjected to Western blot analysis. For experiments using the RIPA buffer, PC12 cells and LMPC microdissected cells were respectively lysed by adding 12 μl of RIPA buffer and 3 μl 5× Laemmli buffer. Cell lysates with RIPA were heat-denatured for 5 min at 105 °C then subjected to Western blot analysis.