Journal of Neuroscience Methods 176 (2009) 172–181

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

NMDA receptor subunit expression in GABAergic interneurons in the prefrontal cortex: Application of laser microdissection technique

Dong Xi[a,b,1], Benjamin Keeler[a,1], Wentong Zhang[b], John D. Houle[a,**], Wen-Jun Gao[a,*]

[a] Department of Neurobiology and Anatomy, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129, United States
[b] Department of Pediatric Surgery, Qilu Hospital and College of Medicine, Shandong University, 250012, China

ARTICLE INFO

Article history:
Received 10 April 2008
Received in revised form 28 August 2008
Accepted 9 September 2008

Keywords:
NovaRed
Laser microdissection
Real-time polymerase chain reaction
Parvalbumin
NMDA receptors
Schizophrenia

ABSTRACT

The selective involvement of a subset of neurons in many psychiatric disorders, such as gamma-aminobutyric acid (GABA)-ergic interneurons in schizophrenia, creates a significant need for in-depth analysis of these cells. Here we introduce a combination of techniques to examine the relative gene expression of N-methyl-D-aspartic acid (NMDA) receptor subtypes in GABAergic interneurons from the rat prefrontal cortex. Neurons were identified by immunostaining, isolated by laser microdissection and RNA was prepared for reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR. These experimental procedures have been described individually; however, we found that this combination of techniques is powerful for the analysis of gene expression in individual identified neurons. This approach provides the means to analyze relevant molecular mechanisms that are involved in the neuropathological process of a devastating brain disorder.

The central nervous system is a complex structure composed of heterogeneous cell types with distinct morphologies and functions. In the neocortex, the inhibitory GABAergic system consists of many different subclasses of interneurons, each having unique phenotypes defined by their morphology, content of neuropeptide and calcium-binding protein (CaBP), electrophysiological property, and synaptic connectivity (Freund and Buzsaki, 1996; Kawaguchi, 1995; Markram et al., 2004). Most importantly, many neurological disorders exhibit cell-type specific damage in the pathophysiological processes of the disease. For example, in schizophrenia, there is selective damage of a subset of interneurons in the corticolimbic system (Akbarian, 1995; Beasley and Reynolds, 1997; Benes and Berretta, 2001; Benes et al., 1991; Guidotti, 2000; Hashimoto et al., 2003; Lewis et al., 2005; Mirnics et al., 2000; Volk et al., 2000; Woo et al., 1998). This damage creates technical challenges for analyzing the relevant molecular mechanisms that are involved in this devastating brain disorder. Biochemical techniques for the study of protein or RNA expression mainly rely on homogenization and extraction of brain regions of 1 mm^3 or larger. These tissue samples are large enough to provide sufficient material to carry out multiple analyses but they unavoidably encompass both affected and unaffected neuronal populations. This could mask the biologically relevant changes presenting in either a limited number of cells or a specific subpopulation of cells. The data obtained by homogenization of such heterogeneous samples are often difficult to reconcile with alterations of specific types of neurons (Murray, 2007; Ruzicka et al., 2007). Therefore, it is preferable to analyze specific cell types when attempting to identify and define biologically important processes.

To achieve molecular analysis of morphologically and phenotypically identified cells, rapid, efficient and accurate methods for obtaining specific groups of cells for further study have been developed. Laser microdissection (LMD) combines microscope-based morphological methods of analysis with a diverse range of very powerful molecular technologies (Curran and Murray, 2005; Espina et al., 2006; Simone et al., 1998). LMD is a relatively new technique with the capability of selectively picking up a brain region/nucleus or a subpopulation of neurons under direct microscopic visualization (Emmert-Buck et al., 1996; Espina et al., 2006; Kubista et al., 2006; Simone et al., 1998). On the other hand, real-time polymerase

Abbreviations: αCaMKII, calcium/calmodulin-dependent protein kinase II alpha; CaBP, calcium-binding protein; CB, calbindin; CD11B, cluster differentiation molecule; CR, calretinin; Ct, threshold cycle; DEPC, diethylpyrocarbonate; DNA, deoxyribonucleic acid; GABA, gamma-aminobutyric acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; LMD, laser microdissection; NMDA, N-methyl-D-aspartate; PFC, prefrontal cortex; PV-ir, parvalbumin-immunoreactive; RNA, ribonucleic acid; ROI, region of interest; RT-PCR, reverse transcription polymerase chain reaction; SNP, single nucleotide polymorphism.

* Corresponding author. Tel.: +1 215 991 8907; fax: +1 215 843 9802.
** Corresponding author. Tel.: +1 215 991 8295; fax: +1 215 843 9802.
E-mail addresses: jhoule@drexelmed.edu (J.D. Houle), wgao@drexelmed.edu (W.-J. Gao).
[1] These authors contribute equally.

0165-0270/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2008.09.013

chain reaction (PCR) is a powerful method for quantification of gene expression based on amplifying specific strands of DNA (Ginsberg et al., 2004; Higuchi et al., 1992; Kubista et al., 2006; Valasek and Repa, 2005).

In order to examine the subunit properties of NMDA receptors and their responses to drug treatment on a subpopulation of interneurons that are selectively damaged in the prefrontal cortex (PFC) of schizophrenia model, we have adapted detailed procedures of rapid RNA preserving immunostaining, LMD, RNA extraction and reverse transcription PCR, electrophoresis, real-time PCR and gene expression analysis. This study represents our initial attempt to detect changes in gene expression in a subpopulation of interneurons in the PFC associated with specific neurological disorders, *e.g.*, schizophrenia, by using the specific captured cells from LMD as the source of RNA for analysis.

1. Methods and materials

1.1. Animals and tissue preparation

Twelve female adult rats (90 days of age) were used in our experiments. The animals were cared for under National Institute of Health (NIH) animal use guidelines, and the experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Drexel University College of Medicine. All rats were anesthetized by an intraperitoneal (*i.p.*) injection of 0.2 ml/kg Euthasol (Henry Schein, Indianapolis, IN) and sacrificed by cervical dislocation. The brain region containing PFC was blocked and immediately frozen in dry ice and stored at −80 °C until further study. Coronal sections were cut at 10 μm at −20 °C in a Vibratome Cryostat (Vibratome, St. Louis, MO). Five to six sections were directly mounted on RNase-free polyethylene napthalate (PEN) foil slides (Leica Microsystems, Wetzler, Germany) and stored at −80 °C in an airtight box to avoid dehydration, whereas adjacent six sections were mounted on gelatin-coated slides and were air dried for Nissl staining with 0.75% cresyl violet solution (Fig. 1A and B). Briefly, gelatin-coated slides mounted with brain sections were rehydrated in distilled water, stained in 0.75% cresyl violet solution for about 30 min, dehydrated in graded ethanol (75%, 95%, 100%, 2× each) and xylene, and coverslipped with DPX Mountant. These sections were used as reference for identification of cortical layers (Fig. 1B).

1.2. NovaRed immunostaining of parvalbumin (PV) in fresh tissue

NovaRed staining uses the ABC kit (Vector Laboratories, Burlingame, CA, USA) to amplify the signal. The procedure is briefly described here and the detailed original protocol can be found in the NovaRed Kit (Vector Laboratories). (1) Thaw the slide mounted with sections at −20 °C for 1 min, then at room temperature for 30 s to attach it with the membrane cohesively. (2) Put the slide into 75% ethanol at −20 °C for 2 min to fix the sections. (3) After rinsing once with diethylpyrocarbonate (DEPC)–phosphate buffer saline (PBS), apply 500 μl mouse anti-PV antibody (1:100 in DEPC–PBS, Chemicon, Millipore, San Francisco, CA, USA) on the sections and then incubate at 40 °C for 8 min. (4) Rinse three times with DEPC–PBS, apply 600 μl universal secondary antibody provided by NovaRed Kit (horse serum and universal antibody 1:48 diluted in DEPC–PBS) and incubate at room temperature for 7 min. (5) Rinse three times with DEPC–PBS, cover the sections with 625 μl ABC (solution A and B 1:24 diluted in DEPC–PBS) (Vector Laboratories) at room temperature for 5 min, and then directly (no rinse) apply 625 μl NovaRed substrate (solution 1 at 1:33 dilution and solutions 2, 3, 4 at 1:50 dilution with DEPC–water) on the slices for 8 min. (6) Transfer the slide into 70% ethanol and then 95% ethanol for 30 s each to dehydrate. (7) Dry the slices at 40 °C for 5 min or at room temperature for 10 min. The whole procedure lasted 38–43 min.

LMD was performed using the Leica LMD system (Leica Microsystems, Bannockburn, IL), which was equipped with 5×, 10×, 20×, and 40× objectives. Prefrontal cortical area can be easily iden-

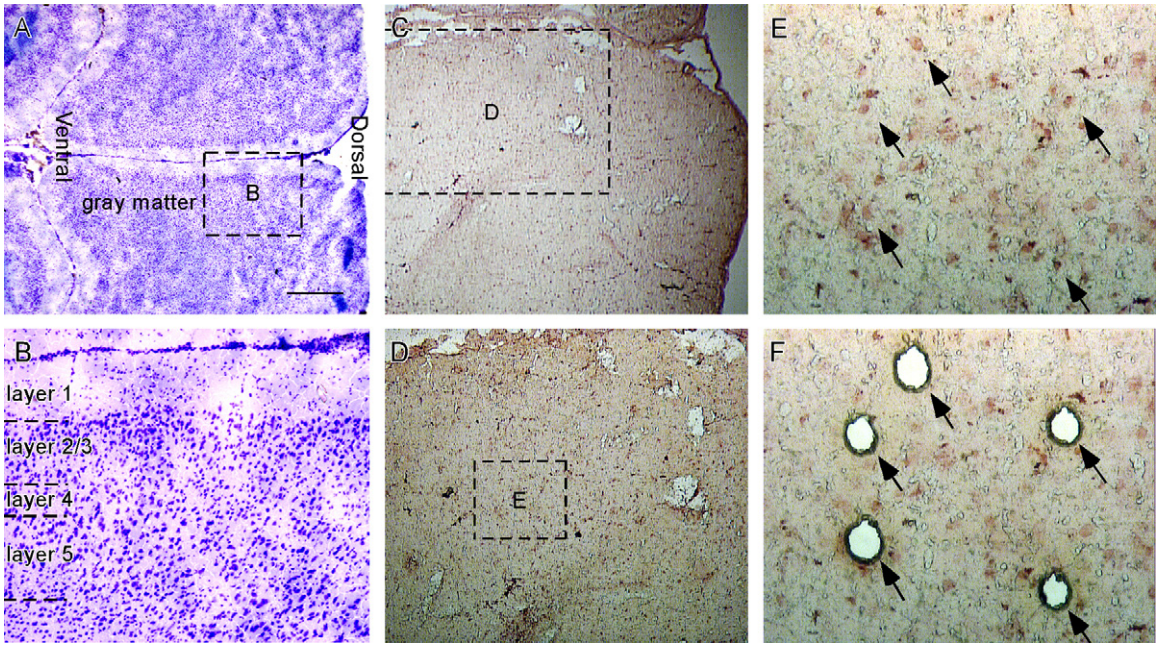

Fig. 1. NovaRed stained PV-ir interneurons in mPFC before and after LMD. (A) Photograph of Cresyl violet-stained frontal cortex with dorsal to the right and ventral to the left of the image. (B) Higher magnification (squared area in A) of Nissl staining showing the laminar architecture of mPFC. Dashed lines delineate cortical layers. (C and D) Photographs of NovaRed-staining at low magnifications showing the area of interest in the mPFC. (E and F) Photographs from (D) (squared area) showing the PV-ir interneurons (arrows point to the cells of interest) before (E) and after (F) laser cut. The PV-ir interneurons were stained in brown/red color. Scale bar in A = 1600 μm for (A), 400 μm for (B and C), 200 μm for (D), and 50 μm for (E and F).