Published in final edited form as:

J Neurosci Methods. 2009 January 30; 176(2): 172–181. doi:10.1016/j.jneumeth.2008.09.013.

NMDA receptor subunit expression in GABAergic interneurons in the prefrontal cortex: application of laser micro dissection technique

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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 micro dissection 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.

Keywords

NovaRed; laser micro dissection; real-time polymerase chain reaction; parvalbumin; NMDA receptors; schizophrenia

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

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Conflict of Interest

The authors claim no conflict of interest.

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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 mm³ 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 micro dissection (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 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.

Methods and materials

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 naphthalate (PEN) foil slides (Leica Microsystems, Wetzlar, 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 minutes, 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).

NovaRed immunostaining of parvalbumin (PV) in fresh tissue

NovaRed staining uses the ABC kit (Vector Laboratories, Burlingame, CA, US) 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 minute, then at room temperature for 30 seconds to attach it with the membrane cohesively. 2) Put the slide into 75% ethanol at -20°C for 2 minutes 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 US) on the sections and then incubate at 40°C for 8 minutes. 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 minutes. 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 minutes, 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 minutes. 6) Transfer the slide into 70% ethanol and then 95% ethanol for 30 seconds each to dehydrate. 7) Dry the slices at 40°C for 5 minutes or at room temperature for 10 minutes. The whole procedure lasted 38 to 43 minutes.

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 identified at magnifications of 5× and 10× with the assistance of Nissl stained sections, as shown in Figure 1C and 1D. Microdissections were performed under 40× objective (Fig. 1E and F), with settings ranged from 8 to 10 in aperture, 30 to 32 in intensity, and 2 to 6 in speed. It is our experience that the tissue processing procedure (NovaRed staining) (see Supplemental Figures 1 and 2) and capture of the cells by LMD are better if completed within one hour to preserve the quality of RNA. At least 100 cells were captured from each slide and immersed in 30 μl lysis solution (RNAqueous Micro-kit, Ambion & Applied Biosystems, Austin, TX, US) (Standaert, 2005). The dissected neurons were processed for RNA extraction or they were stored at -80°C .

RNA extraction, reverse transcription PCR and electrophoresis

RNA was obtained from PFC cells captured from LMD using Ambion RNAqueous Micro-kit (RNAqueous Micro-kit, Ambion and Applied Biosystems, Austin, TX, US) according to the protocol for LMD provided by the manual (Ding and Cantor, 2004)(Supplement Procedure 1). Total RNA was extracted by adding 1.25 volumes of 100% RNase-free ethanol to the cell-lysis mixture. The tRNA, which belongs to small RNA species, is an important component within the extracted product because it is required for the antisense RNA (aRNA) amplification described below. RNA concentration, in the elution solution from the RNAqueous Micro-kit, was routinely measured at 260 and 280 nm wavelength to determine the $\text{OD}_{260/280}$ ratio using the NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, US). The ratios of the isolated RNA located between 1.6 and 2.0 were interpreted as good quality for further processing. In addition, the quality of the isolated RNA was assessed with an Agilent 2100 BioAnalyzer (Agilent Technologies, Inc., Santa Clara, CA) as numerous previous publications reported (Bustin and Nolan, 2004; Kerman et al., 2006; Schroeder et al., 2006). One microliter from each isolated RNA sample was analyzed with RNA Pico LabChips (Agilent Technologies, Santa Clara, CA). The resultant electropherograms were used to determine RNA integrity and concentration (Fig. 2). RNA integrity number (RIN) and 28s/18s ratio could be obtained to assess RNA quality. The RIN was calculated with a proprietary

algorithm (Agilent Technologies, Santa Clara, CA) with 10 being the best and 1 being the worst, whereas 28s/18s ratio was determined by dividing the area under the 28S peak by that of the 18S peak (Schroeder et al., 2006).

Reverse transcription PCR was performed to ensure the specificity of the LMD procedure by using primers for parvalbumin, *i.e.*, to determine whether the dissected neurons were truly PV-immunoreactive (PV-ir) interneurons. Reverse transcription PCR was conducted according to the protocol provided with the Qiagen one-step PCR kit (Qiagen, Valencia, CA, US). PCR products were confirmed by agarose gel electrophoresis. Briefly, 10 μ l of reaction product mixed with 2 μ l loading dye was loaded into gel composed of 30 ml 1% agarose (American Bioanalytical, Natick, MA, US) in 1X Tris-acetate-EDTA buffer (Fisher Scientific, Boston, MA, US) and 1.5 μ l Ethidium bromide (Promega, Madison WI, US). The agarose gel electrophoresis was performed at 110 V for 30 minutes to separate the products according to molecular weight. The PCR products in the gel were visualized with ultraviolet transillumination (Fig. 3). Several genes, including glial fibrillary acidic protein (GFAP, a marker of astrocytes), cluster differentiation molecule (CD11B, which is also known as integrin alpha M, a marker for microglia cells), as well as calcium-binding proteins calbindin (CB) and calretinin (CR), and calcium/calmodulin-dependent protein kinase II alpha (α CaMKII), were tested as negative controls for the neuronal specificity of the LMD samples (Fig. 4).

Antisense RNA (aRNA), synthesis of complementary deoxyribonucleic acid (cDNA), and primer design

The concentration of RNA isolated from PV-ir interneurons is shown in Fig. 5 and the volume obtained from dissection of approximately 100 neurons was about 15 μ l. Because each cell contains only 2 to 100 pg RNA (Bustin and Nolan, 2004), the concentration is too low for one-step real-time PCR. Therefore, two-step real-time PCR, including aRNA amplification and cDNA synthesis, was used to improve the quantity of RNA for detection (Hinkle and Eberwine, 2003; Kannanayakal and Eberwine, 2005). MessageBoosterTM cDNA synthesis kit for qPCR (Epicentre Biotechnologies, Madison, WI, US) was used for amplification of RNA (Shimamura et al., 2004). The aRNA amplification technique can be used to quantify the abundance of mRNA in a very small sample (*e.g.*, single-cell or LMD sample) through linear amplification of poly(A) RNA (mRNA) (Ginsberg and Che, 2004) and mRNA can be reliably amplified (Hemby et al., 2002). Briefly, first strand cDNA was reverse transcribed from total mRNA primed by an T7-Oligo (dT) primer containing the RNA polymerase promoter. Then the cDNA/RNA hybrid produced was digested into small fragments by RNase H, which primes 2nd-strand cDNA synthesis, generating antisense RNA with T7 RNA polymerase. Finally, the amplified aRNA was reverse transcribed into cDNA. The detailed procedure was modified from the protocol provided by the kit by increasing the RNA template from 500 pg to 3 ng (Supplement Procedure 2). The template used for aRNA amplification is 3 ng total RNA isolated from the cells and the final volume collected for each reaction is about 7~10 μ l (Fig. 5). It is necessary that the primers designed for cDNA synthesized by MessageBooster be located within the last 500 bases of the 3'-end of the mRNA. Primers for sequences >500 bases from the 3'-end of the mRNA(s) may give reduced sensitivity according to the instructions for the kit. The use of primer design software is highly recommended (*e.g.*, Primer 3 [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi]) (Schefe et al., 2006) and the boundaries of exons and introns in the sequences were marked in advance [<http://genome.ucsc.edu/cgi-bin/hgBlat>], while the specificity of individual primers were evaluated using BLAST [<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>] (Bustin et al., 2005).

Real-time PCR and normalization of gene expression

Synthesized and preamplified cDNA was diluted to 20 ng/ μ l in RNase-free water or DEPC-water and used as the template for real-time PCR. We chose to use iQTM SYBR[®] Green

Supermix Kit (Bio-Rad, Hercules, CA, US) for our project. Real-time PCR analysis was performed with the primers of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin as internal reference (housekeeping) genes and custom-designed primers of PV and five NMDA receptor subunits (NR1, NR2A, NR2B, NR2C and NR2D). The sequences of these primers are listed in Table 1. Because the threshold cycle (Ct) values of some genes are in the region of 35–39 (see Table 2), it is important for us to run the reaction in 45–50 cycles for a two-step PCR protocol to ensure the complete amplification of all genes (Bustin and Nolan, 2004; Fink et al., 1998). After correct setup and technical quality control of real-time PCR, two parameters for data analysis can be determined: Ct value for each well and PCR efficiency (E) for each gene (Fig. 6) (Fink et al., 1998; Schefe et al., 2006). Relative quantification method $2^{-[\Delta\Delta Ct]}$ was used for normalization of gene expression (Huggett et al., 2005; Livak and Schmittgen, 2001; Yuan et al., 2006). In our experiment, to reduce the inter-sample variability, we repeat each sample 2–4 times and a mean and standard error were obtained for samples from each animal. All Ct values beyond 40-cycle or a reaction exhibiting no exponential rising phase in response curves was excluded from the data analysis. The data were presented as mean $\pm$ standard error and statistic significance was determined with Student's T-test or ANOVA.

Results

NovaRed immunostaining of PV in adult rat PFC and LMD

To identify different types of cortical interneurons and at the same time to preserve the integrity of RNA, we used rapid immunocytochemistry of fresh tissue. Subpopulations of interneurons expressing PV were visualized with NovaRed staining. Figures 1A to 1D show the brain region of the medial PFC at low magnifications. However, only under higher magnification of 40 $\times$ (Fig. 1E) could the PV-ir interneurons be identified. The final staining product of the PV-ir neurons in the cortex is brown/red in color. We used LMD to isolate PV-ir neurons from layers 2/3 to 5 of the medial PFC for analysis of gene expression of NMDA receptor subunits (see Fig. 1E and 1F).

As discussed above, the quality of the isolated RNA was assessed through measuring 260/280 absorbance ratio and RIN. The 260/280 ratio can reach between 1.6 and 2.0, whereas the average RIN was 8.63 ± 0.16 ($n = 9$) with $28S/18S = 1.90 \pm 0.15$ and $18S/\text{baseline}$ value of 7.23 ± 1.17 in a successful experiments (Fig. 2). The RNA sample was considered in good quality when $RIN \geq 7$ and 260/280 ratio was in the range of 1.6 to 2.0 (Kerman et al., 2006).

The quality of the collected PV-ir interneuron RNA

Because only GABAergic interneurons express PV in the neocortex, the LMD captured neurons should contain a high concentration of PV. Figure 3 shows the expression of the RT-PCR amplification of PV derived from the collected samples of six different animals (A–F). Different band densities are attributable to variations in the initial quantity and quality of PV mRNA, PCR reaction conditions, efficiency and/or the volume of individual sample loaded into the gel, but in all samples, there was positive reaction product. Although about 100 immunoreactive cells were isolated with LMD from each sample, it is possible that non-neuronal cells were collected as well since there is close continuity of different cell types in the neuropil. This could then affect the efficiency of the quantitative technique. As shown in Figure 4, GFAP mRNA was expressed in large portions of PV-negative tissue but was not in samples of isolated PV-ir interneurons. This is an example of quality control available with LMD procedures. In contrast, in the PV-ir interneurons, a high density band for PV was observed (Fig. 4E), whereas CD11B, a gene usually expressed in microglia and macrophages and an indicator of inflammation, was negative in both PV-ir and PV-negative tissues.

The specificity was further confirmed with the examination of several other genes, including two similar calcium-binding proteins calbindin (CB) and calretinin (CR), as well as α CaMKII which was only found in cortical pyramidal neurons but not in interneurons (Liu and Jones, 1996). None of these genes were expressed in the LMD captured PV-ir interneurons, whereas α CaMKII was found in PV-negative tissue (Fig. 4F).

Real-time PCR using synthesized cDNA with aRNA amplification as template

Primers of all detected genes (see Table 1), except GFAP and CD11b which work at 60 °C, had been confirmed as working at 55 °C. The concentration of cDNA obtained from MessageBooster (Fig. 5) shows that the amplification from RNA was significantly increased by approximately 400 fold from 3.20 ng/ μ l to 1258.95 ng/ μ l ($p < 0.001$). Fig. 6 shows the representative samples of response curves, including amplification, melting, and standard curves. The Ct values, which were used to calculate the relative expression for all genes tested, ranged from 26.4 to 33.8 (Fig. 6A). The slope of standard curve was -3.35 , indicating a 98.9% efficiency of PCR reaction, whereas the melting curve shown in Fig. 6B is an essential indicator of the quality of PCR products when SYBR green dye is used for GAPDH gene. All of the peaks included in Fig. 6B were around 85°C, demonstrating a single specific PCR product. All of these points indicate that the amplification curve and melting curve were in an acceptable range. If the peak was located lower than 75°C or if there were more than one peak, the PCR reaction would be questionable and troubleshooting should be performed, such as redesigning the primers, changing the annealing temperature and PCR reaction reagents.

Furthermore, we found that the aRNA amplification of extracted RNA from LMD samples is an essential step for real-time PCR (Hinkle and Eberwine, 2003; Kannanayakal and Eberwine, 2005). Figure 6C to F showed the amplification of parvalbumin cDNA derived from the same LMD samples. The 40-cycle reactions showed consistency in the response curves (Fig 6C) and a “single” peak at $\sim 85^\circ\text{C}$ was located in the melting curves (Fig 6D) when synthesized cDNA with aRNA amplification was used as template for the reaction. In contrast, there was no amplification and peak in the unamplified sample (Fig 6E and F).

Relative expression of NMDA receptor subunits in PV-ir interneurons in adult rat PFC

We found that NR1 and several NR2 subunits were expressed in the PV-ir interneurons, with a significantly higher relative mRNA expression of NR2A compared with NR2B subunits ($p < 0.05$; Table 2) (Fig. 7). NR2C subunit was also detected with an average Ct value of 36.3 ± 0.61 , however the NR2D subunit was not present at a detectable level. This result indicates that PV-ir interneurons in the rat PFC differentially express NMDA receptors, with a relative high NR2A/NR2B ratio. To test the hypothesis of NMDA receptor antagonism in an animal model, we applied MK-801 (dizocilpine) in three young adult female rats and used an additional three rats as saline vehicle control. MK-801 is a non-competitive antagonist of NMDA receptors. It has long been used to model schizophrenia because it can produce a range of symptoms remarkably similar to those of schizophrenic patients and can cause a selective decrease of PV-ir interneurons in the forebrain of animal models (Abekawa et al., 2007; Braun et al., 2007; Deutsch et al., 2003; Eyjolfsson et al., 2006; Rujescu et al., 2006). MK-801 (1.0 mg/kg, *i.p.*) or saline vehicle was applied daily for five days (subchronic treatment) (Jackson et al., 2004; Stefani and Moghaddam, 2005). The rats were then sacrificed for testing 24 hours after the last drug administration. We found that subchronic treatment with MK 801 significantly decreased the expression of PV mRNA by 327-fold (107.4 ± 22.6 in control vs. 0.32 ± 0.07 in MK-801, $p < 0.05$). It also significantly decreased the expressions of all NMDA receptor subunits (6.13- fold for NR1, 100.0 ± 44.4 in control vs. 16.3 ± 5.00 in MK-801; 17.7-fold for NR2A, 100.0 ± 33.2 in control vs. 5.66 ± 5.13 in MK-801; and 21.8-fold for NR2B, 99.1 ± 54.1 in control vs. 4.76 ± 4.31 in MK-801; 85.7-fold for NR2C, 102.2 ± 12.1 in control

vs. 1.19 ± 0.50 in MK-801; $p < 0.05$ for all, Fig. 7B). These data were consistent with previous reports (Abekawa et al., 2007; Eyjolfsson et al., 2006; Rujescu et al., 2006).

Discussion

Here we introduce a combination of techniques to examine the expression of mRNA for NMDA receptor subtypes in PV-ir GABAergic interneurons in rat PFC. Although techniques such as rapid immunostaining, LMD, RT-PCR and real-time PCR, have been used elsewhere (Burbach et al., 2003; Espina et al., 2006; Ginsberg et al., 2004; Hemby et al., 2002; Hinkle et al., 2004; Kerman et al., 2006), we found that this combination is very useful for analyzing gene expression in an identified subpopulation of cells.

The GABAergic system in the neocortex consists of many different subclasses of interneurons (Kawaguchi, 1995; Markram et al., 2004). Biochemical markers, such as the calcium binding protein PV, often are used to distinguish different types of interneurons at a cellular level. A subset of PV-ir interneurons in the corticolimbic systems are commonly found to be damaged in the postmortem of schizophrenic patients (Beasley and Reynolds, 1997; Benes and Berretta, 2001; Benes et al., 1991; Lewis et al., 2005; Pierri et al., 1999). This selective vulnerability creates technical challenges for analyzing changes in the expression of DNA, mRNA, and/or proteins in these damaged neurons when examined in the context of an entire brain region. In addition, accumulating studies show that disrupted NMDA receptor function may underlie a cascade of molecular, cellular, and behavioral aberrations associated with schizophrenia (Coyle, 2006; Dracheva et al., 2001; Maxwell et al., 2006; Moghaddam and Jackson, 2003; Mohn et al., 1999; Olney et al., 1999). Postmortem studies demonstrate region-specific abnormal NR subunits in schizophrenia (Kristiansen et al., 2006; Kristiansen et al., 2007), including decreased NR1, NR2A, and 2C in the PFC (Beneyto and Meador-Woodruff, 2008); increased NR2B in temporal cortex (Grimwood et al., 1999), hippocampus (Gao et al., 2000) and thalamus (Clinton and Meador-Woodruff, 2004); and increased NR2D in the PFC (Akbarian et al., 1996). It has been speculated that differential NMDA receptor subunits distributed on interneurons, particularly PV-ir neurons, may be responsible for NMDA receptor dysfunction (Homayoun and Moghaddam, 2007; Javitt, 2004; Lindsley et al., 2006; Olney and Farber, 1995), but direct evidence is still missing. The distributions of NMDA receptor subtypes in cortical interneurons are also unclear (Blatow et al., 2005). Some studies suggested that interneurons lack NMDA receptor-mediated responses (Goldberg et al., 2003; Ling and Benardo, 1995). We propose that the described combination of techniques will provide an important tool to test the hypothesis of NMDA receptor hypofunction in the identified interneurons in animal models of schizophrenia.

Laser micro dissection is a relatively new technique based upon observations of tissue sections with an inverted bright-field light microscope (Emmert-Buck et al., 1996; Espina et al., 2006; Kubista et al., 2006; Simone et al., 1998). It is useful in isolating subpopulations of cells from a heterogeneous tissue section or a cytological preparation of cells from tissue culture (Burbach et al., 2003; Emmert-Buck et al., 1996). Recent reports have combined LMD with quantitative real-time PCR (Burbach et al., 2003; Burbach et al., 2004; Kerman et al., 2006; Prosnjak et al., 2003; Valerie A.M. Vincent, 2002). These have included genome-wide gene expression analyses using DNA microarrays (Hemby et al., 2002), as well as expression analyses of individual genes using RT-PCR (Kamme et al., 2003; Lu et al., 2004; Luo, 1999; Mutsuga et al., 2004; Torres-Munoz et al., 2001; Ye et al., 2003). This combination has made it possible to study the characteristics of mRNA expression in a specific cell population (Burbach et al., 2003). The ability to analyze a single cell type is an important distinction from global and regional assessments of mRNA expression (Ginsberg and Che, 2004; Ginsberg et al., 2004; Hinkle et al., 2004). The LMD-based real-time PCR introduced in this study represents progress in gene detection from tissue to cellular level (Ding and Cantor, 2004; Fink

et al., 1998; Kerman et al., 2006; Mills et al., 2001). Although this combination has been widely used for pathological examination of tumor tissues (Fend et al., 1999), it remains novel for the study of psychiatric disorders. Recently, several studies have reported the use of this combined procedure in the analysis of thalamic nuclei (Byne et al., 2008), Nissl-stained pyramidal neurons in PFC (O'Connor and Hemby, 2007), and DNA methyltransferase 1 (DNMT1) mRNA in layer I cells (Ruzicka et al., 2007) of postmortem brain tissue from schizophrenia patients, but no study has attempted to examine the gene expression in functionally or morphologically identified interneurons (Bahn et al., 2001; Hemby et al., 2002; Kirby et al., 2007; Stephenson et al., 2007).

In our study, we have detected the subunit expressions of NMDA receptor subunits in PV-ir GABAergic interneurons by using NovaRed rapid immunocytochemistry of fresh brain tissue. Our data suggest that PV-ir interneurons in the PFC express several subtypes of NMDA receptors, with a significantly high proportion of NR2A subunits and relatively low NR2B subunits. This result is consistent with a recent study of PV-ir cultured neurons (Kinney et al., 2006) as well as our recent finding with electrophysiological recordings (Wang and Gao, 2007). It is interesting that NR2C subunit is found at a relative high level whereas NR2D subunit is not detected in the PV-ir interneurons. Importantly, all genes tested, including PV and all NMDA receptor subunits, were significantly decreased in an animal model of MK-801 treatment, which is consistent with previous studies (Abekawa et al., 2007; Eyjolfsson et al., 2006; Rujescu et al., 2006). Clearly, this procedure has an advantage of identifying the gene expression of individual subunits that are often unapproachable for electrophysiology and other techniques.

LMD is reported to be compatible with a variety of tissue types, cellular staining methods, and tissue preservation protocols that allow micro dissection of both fresh and archival specimens (Espina et al., 2006). These include Nissl staining (Eltoum et al., 2002; Lachance and Chaudhuri, 2007; O'Connor and Hemby, 2007; Okuducu et al., 2003; Tanji et al., 2001), immunocytochemistry (Burbach et al., 2004; Fassunke et al., 2004; Gjerdrum et al., 2004; Gurok et al., 2007), immunofluorescence (Fink et al., 2000; Mojsilovic-Petrovic et al., 2004), in vivo fluorogold labeling of neurons (Yao et al., 2005), and in situ hybridization (Gjerdrum and Hamilton-Dutoit, 2005). We, however, found that NovaRed staining has advantages of low background, reliable and stable staining, and without the photo bleach problem of immunofluorescence (Burbach et al., 2004). The procedure of NovaRed is also simple and the secondary antibody is universal for both mouse and rabbit primary antibodies. Although several previous studies have described rapid immunofluorescence staining (Fink et al., 2000; Kinnecom and Pachter, 2005; Meguro et al., 2004), we were unable to obtain a satisfactory result. In contrast, subpopulations of interneurons were easily visualized with NovaRed staining. Despite the advantages, special attention should be paid to the RNA quality, primer design, and verification of PCR products to ensure the specificity, efficiency, and reproducibility of the findings. Although RNA degradation is unavoidable during all of these procedures, we found that time of NovaRed staining, LMD procedure, and RNase inhibition play the most critical role in RNA quality [see Fend et al Table 3, (Fend et al., 1999)]. First, all procedures should be performed under strict RNase-free conditions to reduce foreign RNA contamination and use of an RNase inhibitor is recommended in the RNA extraction. Second, the brain slices mounted on slides should be stored in airtight condition to avoid dehydration. Otherwise, NovaRed staining will not work properly. The time is an extremely critical variable when employing LMD for the purpose of RNA analysis (Burbach et al., 2003; Kinnecom and Pachter, 2005). Burbach et al recommended that the LMD procedure should last no longer than 2 hours (Burbach et al., 2003), whereas Kinnecom and Pachter stated that capture sessions longer than 30 min would result in precipitous RNA loss (Kinnecom and Pachter, 2005). We found that the tissue slices had become fragile and RNA is likely degraded after exposing at room temperature for over 30 minutes. It is therefore important to complete NovaRed staining

and LMD cell capture procedures within one hour. Finally, RIN and 260/280 ratio measurement are a necessary step to examine the quality of the isolated RNA. We have succeeded in running a combination of procedures, *i.e.*, from drug treatment of animals, fresh tissue section, rapid immunostaining with NovaRed, LMD, RNA extraction and reverse transcription PCR, real-time PCR and analysis of gene expression for cell-type specific analysis of gene expression. This is certainly an important resource for the elucidation of disease mechanisms and validation of differentially expressed genes as novel therapeutic targets and prognostic indicators, as well as for retrospective clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Mr. George Stradtman for comments on the manuscript.

Funding sources

This study was supported by a grant from Drexel University College of Medicine, a NARSAD young investigator award and NIH R21 grant MH232307 to W-J Gao; and NIH R37 NS26380 to JD Houle. The NIH had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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 micro dissection
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

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Figure 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.

Figure 2.

Measurements RNA integrity number in isolated RNA from a LMD PV-ir sample with Agilent BioAnalyzer. A, gel electrophoresis image for sample exhibited in B. B, electropherogram of RNA isolated from LMD picked PV-ir interneurons. 5S covers the small rRNA fragments (5S and 5.8S rRNA and tRNA), while 18 S and 28 S cover the 18S peak and 28S peak. C, summary graph showing the RIN (8.63 ± 0.16 , $n = 9$) as well as 28/18S ratio (1.90 ± 0.15) and 18S/baseline value (7.23 ± 1.17).

Figure 3. RT-PCR amplification of PV (150 bp) from different samples. Lanes 1 to 6 indicate the cells expressing PV captured from six different animals (A–F). The right lane was the molecular weight marker with 100 bp intervals.

Figure 4.

Gene expressions in PV-ir interneurons versus PV-negative tissues. A and B, photographs showing the PV-ir interneurons (arrows) before (A) and after (B) laser cut. C and D, section of NovaRed staining showing the PV-negative areas (red cycles in C) and the corresponding holes (D) cut as PV-negative tissue sample. Arrows point to the PV-ir interneurons. E, electrophoresis of the three different genes in the PV-ir interneurons and PV-negative tissues. There was no expression of either GFAP or Cd11b in PV-ir cells compared with a positive band of GFAP in PV-negative tissue. F, although calcium/calmodulin-dependent protein kinase II alpha (α CaMKII), which only expressed in cortical pyramidal neurons (Liu and Jones, 1996), was observed in PV-negative control tissue, all of other three genes, including calbindin

(CB), calretinin (CR), and α CaMKII, were not expressed in PV-ir interneurons. Scale bar in B = 50 μ m for A–D.

Figure 5.

Comparison between the concentration of the RNA isolated from LMD-picked cells and the concentration of cDNA synthesized and amplified from the RNA. The DNA concentration was increased about 400 times from 3.20 ng/μl to 1258.95 ng/μl. * indicates the significant difference between two concentrations ($p < 0.001$).

Figure 6.

Real-time PCR response curves. A and B, amplification curves of GAPDH from different dilutions (1:1, 1:4, 1:16, 1:64, 1:256), in which threshold cycle (C_t), the numbers of cycles required to reach threshold, was determined (A). The “single” peak (at ~85°C) exhibited in melting curves (B) suggests a single and specific product. Inset in B, standard curve of the GAPDH derived from (A) showing a correlation coefficient of 0.965 ($R^2 = 0.9306$), slope = -3.35 and PCR efficiency of 98.9%. Efficiency is relevant to slope and the mathematical algorithm is: $(1 + \text{Efficiency})^{-\text{slope}} = 10$. Figures C to F were derived from same LMD sample for PV gene. C and D, response curves (C) and melting curves (D) of 40-cycle reactions using preamplified cDNA of PV as template (same concentration with 6 repetitions). C_t values determined in C ranged from 29.8 to 32.9, and all peaks were located around 85°C in the melting curves (D), indicating the aRNA amplification was successful and specific. E and F, 50-cycle reactions using reverse transcribed cDNA of PV without aRNA amplification. In contrast, there was no amplification and peak in this sample, suggesting that aRNA amplification is an essential step for real-time PCR for LMD sample.

Figure 7.

Relative mRNA expression of NMDA receptor subunits and PV in PV-ir interneurons in normal adult rat PFC versus treatment with MK-801. A, it is clear that the mRNA expression of NR2A subunit is significantly higher than that of NR2B ($p < 0.05$) in the PV-ir interneurons of adult rat PFC (postnatal day 90). Note the high expression of PV and undetectable level of NR2D subunit in PV-ir cells. B, subchronic treatment with MK-801 significantly downregulated the mRNA expression of PV to an almost undetectable level (337.2-fold) and all subunits of NMDA receptors in PV-ir interneurons, *i.e.*, 6.13-fold for NR1, 17.7-fold for NR2A, and 21.0-fold for NR2B ($p < 0.05$).

Table 1

Primers and other information of the genes tested in this study.

Gene	Access #	Forward primer	Backward primer	Product
GAPDH	NM_017008	ccatcccagaccccataac	gcagcgaactttattgatgg	78 bp
β -actin	NM_031144	tgacaggatgcagaaggaga	tagagccaccaatccacaca	104bp
PV	NM_022499	aagagtgcggatgatgtgaag	agccatcagcgtctttgtt	150 bp
NR1	NM_017010	cttctccagccactaccc	agaaagcacccctgaagcac	226 bp
NR2A	NM_012573	aggacagcaaggagcaag	acctaaggatgaccgaaga	174 bp
NR2B	NM_012574	tgagtggggaagagagagagg	atggaacaggaatgggtgga	249 bp
NR2C	NM_012575	gggctcctctggcttctatt	gacaacaggacaggacaca	162 bp
NR2D	NM_022797	cccaaatctcaccatcct	gagaggtgtgtctggggcta	198 bp
GFAP	NM_017009	ccccattccctttcttatgc	atacgaaggcactccacacc	116 bp
CD11b	NM_012711	gaccacacctctgtgtgag	ggctccactttgtctctgt	113 bp
CB	M31178	agggatgtgtctgtctgtgt	catctggctacctcccttg	171bp
CR	X66974	taaaggggtgaaggacagg	gccaccctctttccatctct	157bp
α CAMKII	NM_012920	accatcaacccgtccaaac	atggctcccttcagtttct	152bp

Table 2

Data analysis of real-time PCR where target and internal reference gene are amplified in separate wells

Gene	Number of wells	Average Ct value (mean $\pm$ SE)	Δ Ct = (Average target gene Ct – Average GAPDH Ct) (mean $\pm$ SE)
GAPDH	13	34.2 $\pm$ 0.42	
PV	14	31.2 $\pm$ 0.31	–3.04 $\pm$ 0.52
NR1	13	36.4 $\pm$ 0.43	2.21 $\pm$ 0.60
NR2A	12	35.9 $\pm$ 0.53	1.72 $\pm$ 0.68
NR2B	8	38.0 $\pm$ 0.57	3.82 $\pm$ 0.70
NR2C	5	36.3 $\pm$ 0.61	2.13 $\pm$ 0.62
NR2D	7	Undetectable	

Note: Data analysis of real-time PCR. Seven genes were examined, including the internal reference gene (GAPDH) and target genes (PV, NR1, NR2A, NR2B, NR2C, and NR2D), but only NR2D was at an undetectable level. Average Ct value was calculated from Ct value of individual well. Delta Ct value was calculated as Average Ct value of target gene – Average Ct value of internal reference gene and $SE = (SE_1^2 + SE_2^2)^{1/2}$.