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Microarray analysis of fluoro-gold labeled rat dopamine neurons harvested by laser capture microdissection

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Abstract

The cellular heterogeneity of brain tissue presents a challenge to gene expression profiling of specific neuronal cell types. The present study employed a fluorescent neural tracer to specifically label midbrain dopamine neurons and non-dopamine cortical neurons. The labeled cells were then used to visually guide harvesting of the cells by laser capture microdissection (LCM). RNA extracted from the two populations of harvested cells was then amplified, labeled and co-hybridized to high density cDNA microarrays for two-color differential expression profiling. Many of the genes most highly enriched in the dopamine neurons were found to be genes previously known to define the dopamine neuronal phenotype. However, results from the microarray were only partially validated by quantitative RT-PCR analysis. The results indicate that LCM harvesting of specific neuronal phenotypes can be effectively guided in a complex cellular environment by specific pre-labeling of the target cell populations and underlie the importance of independent validation of microarray results.

Keywords: Microarray; Laser capture microdissection; Dopamine neurons

1. Introduction

The central nervous system (CNS) is composed of a great variety of heterogeneous cell types. In addition to the major categories of neurons, astrocytes, oligodendrocytes and microglia, the neuronal population is further subdivided into a vast complex of phenotypes defined by differential gene expression, connectivity and function. Approaches that employ microarray methods for gene expression profiling of microdissected brain regions (Bunney et al., 2003; Mirnics et al., 2000) unavoidably sample a mixture of multiple cell types. Consequently, expression changes uniquely generated in a specific phenotypic component of the cellular mixture will be diluted by different responses in the other cells.

The ability to harvest individual cells by laser capture microdissection (LCM) (Cornea and Mungenast, 2002; Simone et al., 1998; Wittliff and Erlander, 2002) and to use the RNA extracted from these cells for gene expression profiling represents a major advance in the study of specific CNS neuronal phenotypes. However, effective use of LCM to harvest specific cellular phenotypes requires visual guidance by the distinctive anatomy, morphology or specific labeling of the target cells. Previous studies, by other groups, have used LCM to harvest specific neuronal phenotypes from brain for gene expression profiling.

In one approach, midbrain dopamine neurons were harvested by LCM following immunocytochemical identification of tyrosine hydroxylase (TH) immunopositive cells. RNA extracted from 200 TH-positive cells was amplified, radiolabeled and then used to probe a custom cDNA array of approximately one hundred genes (Backes and Hemby, 2003; Fasulo and Hemby, 2003). The paraformaldehyde tissue fixation required for immunostaining would be expected to cause extensive cross-linking of RNA and the absence of validation

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of the array results by in situ hybridization or quantitative RT-PCR (QRT-PCR) make it difficult to assess the utility of this approach.

In another gene profiling study, 100 cells from each of seven different brain nuclei were harvested by LCM guided by rapid Nissl staining to identify neurons and the known anatomical locations of the target nuclei (Bonaventure et al., 2002). Amplified RNA was then applied to a custom microarray of a few thousand cDNAs and comparisons were made between the different nuclei. For each nucleus, expression of one or two known signature genes was enriched. Further, expression differences between nuclei for four, randomly selected genes were shown to roughly correspond to expression levels determined independently by QRT-PCR and the novel finding of unexpectedly equal expression of a particular single gene across all nuclei was verified by in situ hybridization.

A related study used LCM to harvest relatively large subregions of brain and applied the amplified and labeled RNA derived from these sites to Affymetrix mouse chips for global gene profiling (Zirlinger, 2003). Results from this study found general agreement in gene expression between hand dissected material which did not require RNA amplification and the LCM-derived material which did, indicating that the amplification method preserved the overall transcriptome representation of the tissues. Moreover, in situ hybridization was shown to validate the expression patterns determined by microarray analysis of roughly 60% of 18 selected genes. Other studies have used LCM and gene profiling to analyze gene expression in single cells with promising results (Kamme et al., 2003; Tietjen et al., 2003).

The present study used a neuronal tracing technique to fluorescently label a well-characterized neuronal phenotype, the dopamine neurons of the substantia nigra (SN). Labeling was performed using fluoro-gold, a fluorescent dye taken up by synaptic endings or cut axons and retrogradely transported to neuronal cell bodies (Naumann et al., 2000). A single injection of the dye into the rat striatum has been shown to label cortical neurons in mid-cortical layers via the corticostriatal pathway and dopamine neurons of the substantia nigra (SN), zona compacta via the nigrostriatal pathway (McNeill et al., 1999). Pre-labeling the specific target cell population could direct LCM on frozen sections without subjecting the tissue RNA to destructive fixation protocols. The purpose of the study was to determine whether the labeled cells would effectively guide LCM harvesting and if the RNA extracted and then amplified from these cells would maintain transcriptional representation. The latter was evaluated by performing differential two-color gene expression analysis in which gene expression in fluorescently-labeled cortical neurons was compared to gene expression in fluorescently-labeled dopamine neurons. Gene expression in the dopamine neurons had been previously well-characterized and therefore these cells presented an array of positive control genes to test the validity of the approach. Further, microarray results on selected genes were further evaluated by quantitative RT-PCR.

2. Materials and methods

2.1. Animals and stereotaxic injection

Male Sprague–Dawley rats 250–275 g (Harlan, Indianapolis, IN) were anesthetized with ketamine (75 mg/kg)/xylazine (10 mg/kg) and prepared for stereotaxic surgery according to procedures approved by the Wayne State University Animal Investigation Committee. The rats were placed in a Kopf (Tujunga, CA) stereotaxic instrument and 1 μl of a 5% fluoro-gold (Fluorochrome, Denver, CO) solution was bilaterally delivered to the striatum at 0.2 μl/min, using a CMA microinjection pump (North Chelmsford, MA) through a 26.5 g needle. The stereotaxic coordinates for the injections were +1.0 mm from Bregma, ±3.0 mm from the midline and 5.0 mm down from the top of the cortex; the toothbar was set at 3.3 mm below horizontal zero. Injections of fluoro-gold into the striatum retrogradely label neurons of the SN and cortex (CTX) via extensive projections of the nigrostriatal and corticostriatal pathways (McNeill et al., 1999). Rats quickly recovered from the surgery and were euthanized 5 days post-operatively by anesthesia overdose and transcardial perfusion.

2.2. Tissue preparation

For immunocytochemistry, anesthetized rats were transcardially perfused with 50 ml ice-cold phosphate buffered saline (PBS), followed by 500 ml of 4% paraformaldehyde (PFA). Brains was removed, soaked overnight in 4% PFA at 4 °C, then soaked for 36 h in 20% sucrose at 4 °C, and then blocked, embedded in OCT compound (Sakura, Torrance, CA) and stored at −80 °C. Brains were thawed to −20 °C for cryosectioning at 8 μm on a Leica CM1850 cryostat (Bannockburn, IL) and the sections were mounted on gelatin-coated slides and immunostained for tyrosine hydroxylase (TH) using a rabbit anti-TH antibody (Chemicon, Temecula, CA) diluted 1:250 and a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:200. TH immunoreactivity was visualized with Texas-Red conjugated avidin (Jackson Immunoresearch Labs, West Grove, PA) using an IX70 inverted Olympus (Melville, NY) microscope with mercury-arc illumination and standard filter cube. Images were captured using a high-sensitivity variable integration time color CCD Hitachi video camera (Hitachi Kokusai Electric, Tokyo, Japan).

For LCM, anesthetized rats were perfused with 50 ml of ice-cold PBS, followed by 60 ml of ice-cold 20% sucrose. Brains were then removed, blocked and embedded in OCT and stored at −80 °C until sectioning. Brains were cryosectioned at 14 μm and the sections were placed on uncoated slides frozen face-up on dry ice and stored at −80 °C. Just prior to LCM, slides were thawed to −25 °C, rapidly trimmed of OCT, and dehydrated with 75% ETOH 30–60 s, 95% ETOH 45 s, 100% ETOH 2 min, xylene 5 min and air dried for 10 min. Slides used for the LCM for QRT-PCR analysis