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Single-Cell Gene Expression Analysis: Implications for Neurodegenerative and Neuropsychiatric Disorders*

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Technical and experimental advances in microaspiration techniques, RNA amplification, quantitative real-time polymerase chain reaction (qPCR), and cDNA microarray analysis have led to an increase in the number of studies of single-cell gene expression. In particular, the central nervous system (CNS) is an ideal structure to apply single-cell gene expression paradigms. Unlike an organ that is composed of one principal cell type, the brain contains a constellation of neuronal and noneuronal populations of cells. A goal is to sample gene expression from similar cell types within a defined region without potential contamination by expression profiles of adjacent neuronal subpopulations and noneuronal cells. The unprecedented resolution afforded by single-cell RNA analysis in combination with cDNA microarrays and qPCR-based analyses allows for relative gene expression level comparisons across cell types under different experimental conditions and disease states. The ability to analyze single cells is an important distinction from global and regional assessments of mRNA expression and can be applied to optimally prepared tissues from animal models as well as postmortem human brain tissues. This focused review illustrates the potential power of single-cell gene expression studies within the CNS in relation to neurodegenerative and neuropsychiatric disorders such as Alzheimer's disease (AD) and schizophrenia, respectively.

KEY WORDS: Alzheimer's disease; cDNA microarray; cholinergic basal forebrain; dopamine receptors; expression profiling; protein phosphatases; RNA amplification; schizophrenia; single-cell microaspiration.

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Abbreviations (note the use of the NCBI-Unigene annotation): 3Rtau, three-repeat tau; 4Rtau, four-repeat tau; ACT, alpha-1-antichymotrypsin; ACTB, beta-actin; AGER, advanced glycosylation end product–specific receptor; APOE, apolipoprotein E; APP, amyloid-beta precursor protein; arc, activity regulated cytoskeletal-associated protein; BAX,

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BCL2-associated X protein; BCL-2, B-cell lymphoma 2; CAMKII, calcium/calmodulin-dependent protein kinase II, alpha; CBP, CREB binding protein; CDK2, cyclin-dependent kinase 2; cdk5, cyclin-dependent kinase 5; cdk5R1, cyclin-dependent kinase 5, regulatory subunit 1 (p35); c-fos, cellular oncogene fos; c-jun, jun oncogene; CREB, cAMP responsive element binding protein; CREM, cAMP responsive element modulator; CTSD, cathepsin D; DAT, dopamine transporter; DRD1, dopamine receptor D1; DRD2, dopamine receptor D2; DRD3, dopamine receptor D3; DRD4, dopamine receptor D4; DRD5, dopamine receptor D5; EAAT3, excitatory amino acid transporter 3; ERK1, extracellular signal-regulated kinase 1 (p44); ERK2, extracellular signal-regulated kinase (p42); FAK, focal adhesion kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBL, G protein beta subunit-like; GFAP, glial fibrillary acidic protein; GNG1, G protein, gamma 1; GNG2, G protein, gamma 2; GRIA1, AMPA1; GRIA2, AMPA2; GRIA3, AMPA3; GRIA4, AMPA4; GRIA5, kainate 5; GRIA6, kainate 6; GRIA7, kainate 7; GRIN1, *N*-methyl D-aspartate 1(NMDAR1); GRIN2A, NMDAR2A; GRIN2B, NMDAR2B; GRIN2C, NMDAR2C; GNAI1, guanine nucleotide binding protein (G protein), alpha inhibiting 1; GNAI2, G protein, alpha inhibiting 2; GNAS1, G protein, alpha stimulating activity polypeptide 1; GNAQ, G protein, q polypeptide; GNAZ, G protein, alpha z polypeptide; GRX, glutaredoxin; GSK-3B, glycogen synthase kinase-3 beta; HSPG, heparan sulfate proteoglycan; IL-1, interleukin 1; IL-6, interleukin 6; junB, jun B proto-oncogene; junD, jun D proto-oncogene; MAP1b; microtubule-associated protein 1b; MAP2, microtubule-associated protein 2; NF-H, neurofilament heavy subunit; NF-L, neurofilament light subunit; NF-M, neurofilament medium subunit; NTRK1, low affinity nerve growth factor receptor (trkA); NTRK2, trk(B); NTRK3, (trkC); p75, low affinity nerve growth factor receptor; PP1A, protein phosphatase 1 catalytic subunit, alpha isoform; PP1G, protein phosphatase 1, catalytic subunit, gamma isoform; PP2AB, protein phosphatase 2, regulatory subunit A, beta isoform; PP2AC, protein phosphatase 2, catalytic subunit, alpha isoform; PSD95, postsynaptic density protein 95; PSEN1, presenilin 1; PSEN2, presenilin 2; SNAP-23, synaptosomal-associated protein, 23 kDa; SNAP-25, synaptosomal-associated protein, 25 kDa; SNCA, alpha synuclein; SNCB, beta synuclein; SOD1, superoxide dismutase 1; SYB, synaptobrevin; SYB2, synaptobrevin 2; SYN1, synapsin 1; SYNGR1, synaptogyrin 1; SYNGR3, synaptogyrin 3; SYP, synaptophysin; SYT, synaptotagmin; TR1A, tumor necrosis factor receptor superfamily member 1A; TUBB, beta-tubulin; VAMP2, vesicle-associated membrane protein.

EXPRESSION PROFILING USING FIXED AND FROZEN TISSUE SOURCES

Expression profile analyses of postmortem human brain tissues and optimally prepared fixed animal model tissues is an experimental scheme that is quite appealing due to significant amounts of archived brain tissues available from research laboratories and brain banks. At present, there is no universally accepted method for optimal tissue fixation for molecular-based studies. In addition to using fresh-frozen samples, RNA can be extracted from brains fixed in precipitating fixatives such as 70% ethanol buffered with 150 mM sodium chloride as well as cross-linking fixatives such as paraformaldehyde and 10% neutral-buffered formalin (1–6). Variables including antemortem characteristics, agonal state, fixation conditions, and length of storage should be considered prior to initiating expression profiling studies (7–10).

One method used to determine presence detection and quality of RNA species in tissue sections is acridine orange (AO) histofluorescence. AO is a fluorescent dye that intercalates selectively into nucleic acids (11,12) and has been used to detect RNA and DNA in brain tissues (13,14). AO histofluorescence detects the presence of RNA species in normal neurons that are contrasted by the pale background of adjacent neuropil and white matter lacking abundant nucleic acids. The majority of AO-labeled profiles within the CNS are predominantly neuronal in origin, as glial populations in human brain do not contain appreciable levels of AO histofluorescence (15–17). Although several other methods have been proposed to assess the integrity of RNA species in postmortem tissues (i.e., measuring brain pH or gel electrophoresis of brain extracts), the direct demonstration of the presence of RNA in tissues using AO histofluorescence increases confidence that the tissues to be examined by expression profiling are suitable for this purpose. Thus, AO histofluorescence provides a convenient and reliable method for RNA detection *in situ* and can be performed on tissue sections adjacent to those designated for single-cell gene expression analysis. In addition, RNA sequestration has been observed in senile plaques (SPs) and neurons that bear neurofibrillary tangles (NFTs) in the brains of patients with Alzheimer's disease (AD) and related disorders using AO histofluorescence (4,15,16).

REGIONAL DISSECTION AND GENE EXPRESSION

Global or regional expression of gene levels is a paradigm used widely in the brain due to relatively large amounts of RNA that can be extracted and used for a variety of downstream genetic analyses in animal models including studies of amyloid overexpression (18), experimental Parkinsonism (19,20), hippocampal cytoarchitecture (21), and stress on the hypothalamus (22). Regional analyses of gene expression can also be performed on postmortem human brain tissues, as evidenced by reports on several neurodegenerative and neuropsychiatric disorders including AD (23,24), amyotrophic lateral sclerosis (25–27), multiple sclerosis (28,29), and schizophrenia (30–33). One advantage of regional gene expression analysis is that, in most cases, extracted RNA is sufficient to generate significant hybridization signal intensity for cDNA arrays without additional Polymerase chain reaction (PCR)–based or linear RNA amplification

protocols. However, an obvious disadvantage is the lack of single-cell resolution, as multiple subpopulations of neurons, noneuronal cells, vascular elements, and epithelial cells will contribute to the total input RNA source.

ACCESSION OF SINGLE CELLS FROM TISSUE SECTIONS

A critical factor that helps to determine the specificity of sophisticated genetic testing methods is purity and precise acquisition of identified cells. Specifically, single-cell and/or single-population molecular analysis necessitates accurate, nondestructive isolation of cells from optimally prepared tissue sections (34). Two widely used microdissection methodologies are laser capture microdissection (LCM) and microaspiration. LCM employs a high-energy laser source that separates desired cells from the rest of a tissue section and facilitates transfer of the identified cells to a microfuge tube for genetic analysis (35,36). Single cells as well as hundreds of cells can be acquired via LCM in a relatively short time. Individual cells and pooled populations of similar cell types are visualized by immunocytochemical and/or histochemical procedures for optimal identification of specific cells of interest (3,34). RNA, DNA, and protein extraction methods can be performed on microdissected cells (37–40), although LCM is primarily used for RNA extraction and subsequent cDNA microarray analysis. Control experiments for single-cell analysis include no input of RNA (no template control) and RNase pretreatment of tissue sections prior to LCM of specific cells (2,3,34).

Microaspiration entails visualizing individual cells or their processes using an inverted microscope workstation connected to a micromanipulator and microcontrolled vacuum source on an air table. Individual cell(s) are patched onto using a microelectrode and excised. Microaspiration results in accurate dissection of the neurons of interest with minimal disruption of the surrounding neuropil (1,2,5,6,41). Thus, microaspiration enables precise dissections of single elements (i.e., individual neurons, neuropil, and dendrites). Single cells can be used alone or pooled with other cells for qPCR and/or RNA amplification and subsequent microarray analysis. The process of microaspiration is more painstaking than LCM, and provides potentially more accurate dissection of single cells. However, LCM has much greater throughput capabilities than microaspiration. Essentially, the experimental design of each study dictates what microdissection technique is best suited for each specific application.

qPCR USING SMALL SAMPLE INPUT SOURCES

The quantity of total RNA harvested from a single cell, estimated to be approximately 0.1–1.0 picograms, is below the level of sensitivity for standard RNA extraction procedures (42–44). Therefore, alternative methods of analysis have been used including semiquantitative *in situ* hybridization histochemistry (ISH), quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR), and linear RNA amplification. ISH is a useful technique for presence detection and estimation of relative abundance of specific mRNA transcripts within single cells (7,45). Due to technical considerations including limited sensitivity and relative inability to assess multiple mRNAs simultaneously, ISH is ideally suited for regional gene expression pattern assessments and is routinely used as a validation technique for microarray studies (2,22, 33,46). Standard RT-PCR can amplify genetic signals from small tissue samples. However, PCR-based methods tend to amplify abundant genes over rare genes and may distort quantitative relationships among gene populations due to exponential, nonlinear amplification (8,47). Real-time qRT-PCR lessens concerns regarding linear amplification and obviates the need for gel electrophoresis and the use of radioactivity for PCR quantification. Two basic tenets enabled discovery and subsequent development of real-time qPCR. First, DNA polymerases including *Taq* polymerase possess 5′ to 3′ exonuclease activity (48). Second, the principle of fluorescence resonance energy transfer (FRET) (49) can be applied to double-labeled oligonucleotide probes (e.g., PCR and/or RT-PCR primers). In a double-labeled oligonucleotide probe system (ideal for qRT-PCR), FRET occurs when fluorescence energy of a reporter dye is quenched by close proximity of the quencher dye. Thus, fluorescence signal emission occurs only upon cleavage of the probes (i.e., amplification of a specific PCR product during a PCR cycle). A variety of dye chemistries are currently being exploited in qRT-PCR systems including hydrolysis probes (e.g., TaqMan), molecular beacons (ideal for detecting point mutations), and double-stranded DNA binding dyes (e.g., SYBR green) (50).

RNA AMPLIFICATION OF SINGLE CELLS AND POPULATIONS

RNA amplification procedures are now routinely used to amplify input RNA to increase signal detection for microarray analysis. These procedures essentially entail *in vitro* RNA transcription using a double-stranded

(ds) cDNA template to amplify genes in a linear manner (42,51,52). RNA amplification methods preserve the original quantitative relationship(s) in an amplified gene population, thus facilitating downstream quantitative analysis. Amplified antisense RNA (aRNA) synthesis amplifies genetic signals from limited amounts of fresh, frozen, or fixed tissues and cells (1,2,5,6,51,52), and various aRNA reagent kits are available from several companies. To enable a robust *in vitro* RNA transcription reaction for subsequent genetic analyses, a ds cDNA template requires a functional transcriptional promoter sequence. Conventional RNA amplification procedures use a bacteriophage transcriptional promoter sequence (e.g., T7, T3, or SP6) to the 3′ end of first-strand cDNA, resulting in transcripts with antisense orientation. Aspects of the current RNA amplification strategies that would benefit from innovative improvements include increasing the efficiency of second-strand cDNA synthesis and allowing for flexibility in the placement of bacteriophage transcriptional promoter sequences.

A new methodology has been developed in our laboratory to amplify RNA from minute amounts of starting material (3,42). Specifically, the method, termed terminal continuation (TC) RNA amplification, enables an efficient means of second-strand cDNA synthesis using a two-primer system. In addition, an RNA synthesis promoter is attached to the 3′ and/or 5′ region of cDNA using the TC mechanism (3,42). The orientation of amplified RNAs is "antisense," or a novel "sense" orientation. One round of TC RNA amplification is sufficient for downstream genetic analyses (3,42). TC RNA amplification is used for many downstream applications including gene expression profiling, cDNA microarray analysis, and cDNA library/subtraction library construction. Synthesized sense TC amplified RNA can also be used as a template for *in vitro* protein translation and proteomic applications.

cDNA ARRAY ANALYSIS

cDNA array technology enables relative quantitative assessment of multiple (e.g., dozens to thousands) genes simultaneously from one tissue sample. Synthesis of cDNA microarrays entails adhering cDNAs, expressed sequence-tagged cDNAs (ESTs), or oligonucleotides to solid supports such as glass slides, plastic slides, or nylon membranes (53,54). A parallel technology uses photolithography to adhere oligonucleotides to array media (55). Expression profiling is performed by extracting total RNA or mRNA from sample tissues, amplifying the RNA if necessary, labeling the RNA probe, and hybridizing the labeled RNA to microarrays. Arrays are washed to remove nonspecific background hybridization signals and imaged using a phosphor imager or a laser scanner for radioactive probes and fluorescent probes, respectively. Quantification of hybridization signal intensity is performed to assess the relative signal intensity of each cDNA, EST, or oligonucleotide on the array platform. Gene expression is then quantitated using univariate statistics and informatics software that enables large volumes of coordinate analyses. Computational analysis is critical due to the large number of data points that are generated from a single assay. One caveat of cDNA array analysis is that coordinate changes in proteins encoded by the genes of interest are common, but not absolute (56).

SINGLE-CELL ANALYSIS IN AD: NEUROFIBRILLARY TANGLES

The pathogenesis of neurofibrillary tangles (NFTs) in AD and related disorders is poorly understood and is being investigated through a variety of histopathological- and biochemical-based paradigms. We hypothesize that alterations in the expression of specific mRNAs may reflect mechanisms underlying the formation of NFTs and their consequences in affected neurons. To this end, the relative abundance of multiple mRNAs is assessed in NFT-bearing versus normal CA1 neurons isolated from 6-μm-thick ethanol-fixed paraffin-embedded sections of hippocampus from AD brains and neuropathologically confirmed normal controls. The brains accessed for these studies are provided by the Center for Neurodegenerative Disease Research at the University of Pennsylvania School of Medicine (1,2). Each brain is confirmed to have abundant cytoplasmic RNAs by AO histofluorescence prior to use in the expression profiling studies (15,16).

Single-cell RNA amplification is performed in combination with high-density (>18,000 ESTs) and custom-designed (approximately 120 cDNAs/ESTs relevant to neuroscience) array platforms. A correspondence of 85% between the two array platforms is observed (73/86 cDNAs/ESTs) in terms of the direction (e.g., down-regulation, up-regulation, or no change) of specific genes with dual representation (1). For example, all five ESTs linked to protein phosphatase 2A (PP2A) subunit PP2A-Aβ on the high-density microarray are down-regulated in NFT-bearing neurons, consistent with a similar level of relative down-regulation observed on the custom-designed cDNA array platform (1). Relative to normal CA1 neurons, those harboring NFTs in AD brains showed significant reductions in several classes of mRNAs that are known to encode proteins implicated in AD neuropathol-

ogy including protein phosphatases/kinases, cytoskeletal elements, synaptic-related markers, glutamate receptors (GluRs), and dopamine (DA) receptors (1,57). A summary of representative mRNA changes is presented in Fig. 1A. Total hybridization signal intensity is down-regulated in single AD NFT-bearing neurons compared to normal CA1 neurons by approximately 28%–36%, consistent with previous semiquantitative studies of polyadenylated mRNA expression in AD (58,59). Amyloid-β protein precursor (APP) mRNA is decreased approximately twofold on both high-density and custom-designed cDNA arrays, potentially indicating that NFT-bearing neurons express less APP mRNA in affected regions of the AD brain. Furthermore, a two - to fourfold decrease in the expression of the mRNAs for DA receptors D1–D5 and the DA transporter is observed in NFT-bearing neurons in AD versus non–tangle bearing neurons in control brains (1,57). These observations are consistent with data showing decreased D2 receptor binding in the AD hippocampus (60,61), and they underscore the advantages of single-cell mRNA analyses because antibodies and ligand-based studies have not been able to discriminate unequivocally between DA receptor subtypes. In addition, a reduction in gene expression levels of synaptic-related markers is observed, implicating NFTs in synaptic damage to affected neurons. Synaptic-related markers that are down-regulated include synaptophysin (2.2-fold), synaptotagmin (2.0-fold), synapsin I (2.5-fold), α-synuclein (2.5-fold), and β-synuclein (2.3-fold) (1).

Analysis of the high-density cDNAs microarrays revealed several ESTs are up-regulated in AD CA1 neurons with NFTs. However, out of the 50 most up-regulated ESTs, only one is linked to a protein of known cellular function: cathepsin D (1). Furthermore, an approximate twofold up-regulation of cathepsin D is observed on the custom-designed array platform, and paired helical filament tau (PHFtau) positive NFTs co-localize with increased cathepsin D immunoreactivity in the same CA1 neurons (1,57). Up-regulation of the acid hydrolase cathepsin D is consistent with a growing body of literature indicating that activation of the lysosomal protease system is an early alteration in AD and may prove to be an important biomarker of the disease (62–64). These data illustrate an experimental strategy to employ both high-density and custom-designed cDNA microarrays and single-cell RNA amplification to identify alterations in the expression levels of numerous transcripts in AD NFT-bearing neurons compared to normal control CA1 neurons. Future studies include assessments of early stages of AD and mild cognitive impairment to delineate the earliest changes that occur within the brain during the development of dementia.

SINGLE CELL ANALYSIS IN AD: SENILE PLAQUES

The polypeptide composition of NFTs and senile plaques (SPs) has been characterized within AD brain (57,65,66). However, little data exists on the nonproteinaceous components of these lesions. The presence of RNA species in NFTs and SPs is evaluated using AO histofluorescence alone or in combination with thioflavine-S (TS) staining and immunohistochemistry in AD brains and related neurodegenerative disorders that display NFTs and SPs (15,16). Quantitative analysis of double-labeled sections demonstrates approximately 80% of TS-positive NFTs are AO-positive, whereas approximately 55% of TS-stained SPs also contain AO labeling (15,16). Thus, these data demonstrate the presence of RNA species in a significant population of NFTs and SPs. The observed sequestration of RNA SPs in AD and related disorders prompted single-cell analysis of amyloid-β protein (Abeta)-immunolabeled SPs in sections of AD hippocampus. The expression profile of SPs is compared to individual CA1 neurons and surrounding neuropil of control brains using single-cell RNA amplification coupled with custom-designed cDNA arrays (2). Results indicate that SPs harbor two distinct populations of gene expression levels: high abundance genes including APP, tau, bcl-2, bax, PP subunits, and several GluRs, and low abundance genes including neurofilament subunits and glial-enriched mRNAs (2). A molecular fingerprint of SPs obtained from the CA1-subiculum region of AD brains is presented relative to CA1 neurons in normal brains (Fig. 1B). Additionally, a representation of glial-associated mRNAs enriched in neuropil (NP) microaspirated from AD brains is shown relative to SPs (Fig. 1C). The presence of mRNA species in extracellular SPs is validated further by combined ISH and TS using a probe against cyclic AMP response element binding protein (CREB) as well as PCR for APP isoforms from individual SPs (2). These data indicate that multiple mRNA species are found in individual, extracellular SPs of the AD hippocampus by the combined use of single-cell expression profiling, PCR, and ISH methods. Moreover, the expression profile of mRNAs amplified from SPs is predominantly neuronal. These observations suggest that SPs sequester the remnants of degenerating and/or dying neurons and their processes during their formation. Microglial cells have been detected within SPs, and astrocytosis occurs around SPs (15,67,68), but relatively low levels of glial-derived mRNAs are seen in SPs, which may also reflect a lower abundance of RNAs in glial cells (17).

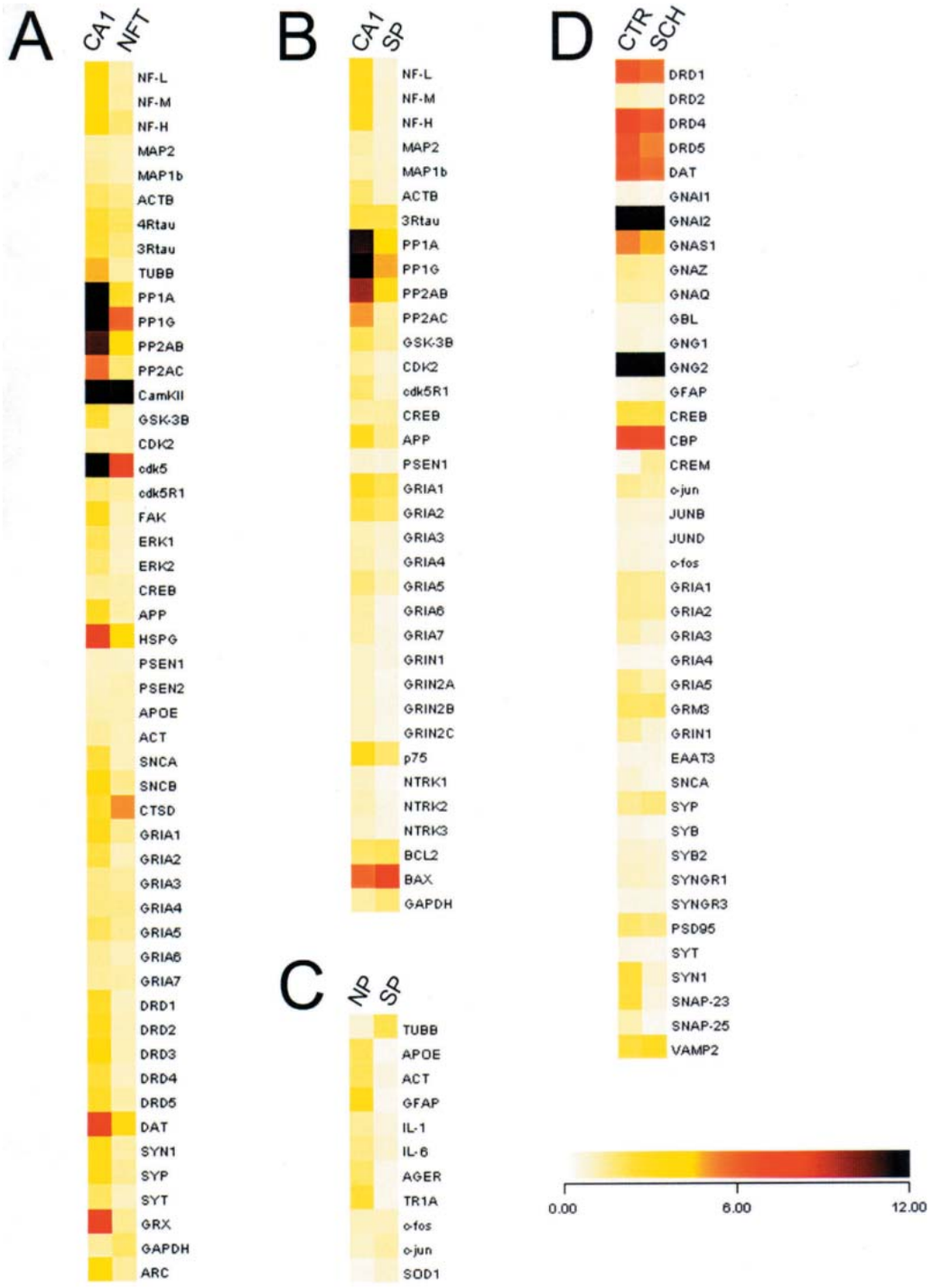

Fig. 1. Dendrograms illustrating relative expression levels of representative transcripts for several single-cell expression profile paradigms. (A) A comparison of expression profiles from normal control CA1 pyramidal neurons and NFT-bearing CA1 neurons in AD. (B) Expression profiles garnered from CA1 pyramidal neurons from normal control brains and SPs from AD brains. (C) A series of glial-associated mRNAs illustrate the paucity of glial mRNA representation in SPs from AD brains relative to adjacent neuropil (NP) accrued from the CA1-subicular region of AD brain. (D) Assessment of representative transcripts obtained from individual layer II entorhinal stellate cells from normal control subjects (CTR) and schizophrenics (SCH).

SINGLE-CELL ANALYSIS IN AD: CHOLINERGIC BASAL FOREBRAIN (CBF) NEURONS

CBF neurons supply the majority of cholinergic fibers to the cerebral cortex and hippocampal formation and are key anatomic substrates of memory and attention systems in the brain (69–71). CBF neurons of the nucleus basalis (NB) are selectively vulnerable in AD (72,73), and CBF neuron degeneration correlates with disease duration and cognitive decline (74,75). However, the molecular mechanisms(s) associated with CBF cytopathology and cellular dysfunction is unknown. Single-cell RNA amplification and custom-designed cDNA array technology are employed to examine expression of several functional classes of mRNAs found in anterior NB neurons from normal aged and AD subjects (4,76). Similar to observations in NFT-bearing CA1 neurons (1), a down-regulation of synaptophysin, synaptotagmin, and PP1 subunit mRNAs, and an up-regulation of cathepsin D mRNA is observed in AD compared to normal control CBF neurons (4,76). Single-cell analyses provide clues to the molecular pathogenesis of neurodegeneration within the NB. Because CBF neurons are also an early target for NFT formation (77,78), a double-label PHFtau and choline acetyltransferase immunocytochemistry paradigm combined with single-cell mRNA analysis is a future direction that may help elucidate early alterations in these selectively vulnerable populations within the basal forebrain. Finally, the presence of extracellular SPs in the terminal fields of cholinergic innervation within the neocortex and hippocampus of AD brain suggests that alterations in the metabolism and transport of APP in CBF neurons may play a role in plaque formation (79,80), as evidenced by axotomy paradigms in mouse models of amyloid deposition where reduction of amyloid accumulation is observed on the side ipsilateral to the lesion (81–83).

SINGLE-CELL ANALYSIS IN SCHIZOPHRENIA

Schizophrenia is a chronic psychiatric illness affecting approximately 1% of the general population. Clinical manifestations appear in late adolescence to early adulthood, including a mixture of positive and negative symptoms. The etiology of schizophrenia remains unknown, although several lines of evidence implicate multigenic predisposition, neurodevelopmental alterations, and environmental events as potential causative factors (84–86). One brain region affected in schizophrenia is the temporal lobe, including the hippocampus and entorhinal cortex. Both neuroimaging studies and neuropsychological testing of schizophrenics indicate that the temporal lobe is affected (87,88). Upon pathologic examination, however, relatively little neurodegeneration is observed in schizophrenic brains (89,90). The absence of frank neuropathology is a common difficulty in identifying molecular substrates for neuropsychiatric disorders due to the lack of hallmark pathologic lesions, such as those observed in neurodegenerative disorders including AD, Parkinson's disease, and Lewy body dementia. Nonetheless, the strategic location of entorhinal cortex stellate cells and hippocampal pyramidal neurons makes them excellent candidates for probing disease-related differences in gene expression associated with schizophrenia. These data obtained from pyramidal neurons in neuropsychiatric disorders also provide a disease control database for neurodegenerative disorders where neuropathological changes are readily discernible within the hippocampal formation.

Postmortem regional studies of prefrontal cortex using cDNA microarrays have observed consistent alterations in several classes of relevant transcripts including synaptic-related markers, G-protein–coupled signaling molecules, and oligodendrocyte-related markers (9, 30–33, 46). These regional studies provide an exciting molecular fingerprint of another important affected brain region in schizophrenia. However, these expression profiles cannot delineate alterations that occur selectively within affected neuronal populations from adjacent neuronal, noneuronal, epithelial (e.g., ependymal cells), and vascular elements.

Single-cell gene expression profiling is performed on stellate cells from layer II of entorhinal cortex obtained from the brains from eight schizophrenics and nine age-matched neurologically normal controls (5). Postmortem brain tissue is obtained from poor-outcome, elderly, chronically hospitalized patients from the established brain collection of the Mental Health Clinical Research Center on Schizophrenia at the University of Pennsylvania (5). Control subjects without history of neurological or major psychiatric illness are obtained via the Center for Neurodegenerative Disease Research at the University of Pennsylvania (1,6,57). Two high-density cDNA microarray platforms containing approximately 18,240 and 9,710 genes are used in addition to custom-designed cDNA arrays containing approximately 48 ESTs/cDNAs that are demonstrated to be regulated on the high-density platforms. Results indicate significant differences in mRNA expression of various G-protein–coupled receptor signaling transcripts, GluR subunits, synaptic-related markers, and other mRNAs between schizophrenics and control stellate cells (5). Expression profiling using custom-designed arrays reveals significant decreases in G-protein mRNAs including GNAS1, GluRs including GluR3, GluR5, and

NMDAR1, and synaptic-related markers including synaptophysin, SNAP-23, and SNAP-25 (5), providing a secondary screen for validation of gene expression level changes on the high-density array platforms. The aforementioned single-cell analysis displays some similarity with regional microarray studies, particularly in classes of transcripts involved with signaling, including G-protein–coupled molecules and synaptic transmission (5,31,33). In summary, our study applies a broad-scale functional genomics approach for determining molecular correlates of schizophrenia at the level of individual neurons. These results may be influenced by the age of the sample population, medication history, and the constellation of clinical manifestations of schizophrenia. Understanding the orchestrated expression of dozens to hundreds of genes simultaneously in a human disease with a multigenic etiology and the potential complication of environmental involvement seen in a neuropsychiatric disorder such as schizophrenia provides insight into the molecular pathogenesis of the disease process and may ultimately provide new targets for pharmacotherapeutic interventions.

SINGLE-CELL ANALYSIS AND AGING: DOPAMINE RECEPTOR EXPRESSION

One of the advantages of using single-cell gene expression is that classes of transcripts can be compared across a variety of experimental and disease conditions in discrete cell populations (3,34,52). A paradigm that is quite intriguing is aging, particularly within cell types that are vulnerable to neurodegeneration in late-onset progressive neurodegenerative disorders as well as those implicated in neuropsychiatric disorders (91,92). For example, age-related decline in dopamine (DA) receptor levels is observed in regional brain studies of animal models and human postmortem tissues (93–96). To evaluate potential age-related alterations in DA receptor subtypes in specific cell populations of the hippocampus and entorhinal cortex, single-cell RNA amplification is combined with custom-designed cDNA array analysis to evaluate effects of aging on D1–D5 dopamine receptor mRNA expression levels in hippocampal CA1 pyramidal neurons and entorhinal cortex layer II stellate cells from normal control postmortem human brains aged 19–95 years old (6). Results indicate a significant age-related decline for all five (D1–D5) DA receptor mRNAs in CA1 pyramidal neurons [6]. Percent decline per decade for each receptor subtype is 5.2%, 5.0%, 11.2%, 4.7%, and 5.0%, respectively (6). Down-regulation of DA receptor subtypes appears to be relatively selective, as no age-related decrement in other mRNAs is observed in CA1 pyramidal neurons including the cytoskeletal elements β-actin, three-repeat (3R) tau, and four-repeat (4R) tau (6). In contrast, no significant changes in DA receptor subtype expression are observed in stellate cells across the same cohort. Alterations in hippocampal DA function impact memory and cognitive functions (97,98). Breaches in functional integrity of the hippocampal DA neurotransmission correlates with pathophysiology of neurodegenerative disorders including AD (1,60,61) and neuropsychiatric disorders including schizophrenia (86,99). Deficits in DA systems also may contribute to cognitive decline associated with normal aging (100–102). For example, several studies report age-related decreases in dopamine transmission in temporal neocortex and hippocampus of aged rats and humans (103–106). Our single-cell studies extend and confirm previous observations at the regional/binding site level to include cell-specific localization of DA receptors (6). In summary, senescence may be a factor responsible for cell-type–specific down-regulation of DA receptor gene expression in a circuit crucial for learning and memory that is also vulnerable to pathological changes in neurodegenerative and neuropsychiatric disorders.

CONCLUSIONS

This focused review illustrates the power and potential of analyzing single populations of cells and pathological lesions within the postmortem human brain using RNA amplification coupled with cDNA microarrays and qPCR-based analyses. Our laboratory is slowly accruing a database of expression profiles that will enable a coordinated comparison of molecular fingerprints from a myriad of specific cell types that are vulnerable to neurodegeneration in several neurodegenerative and neuropsychiatric disorders. Parallel expression profiling experiments are also being conducted in relevant animal models to evaluate further and elucidate mechanisms that underlie pathological changes within specific cell types in the CNS.

The application of cDNA microarray technology has generated significant interest across disciplines and spans a multiplicity of biological systems. However, the brain remains a difficult organ to study, in part due to the nuclear, laminar, and cellular heterogeneity of brain regions and cell types (107,108). Thus, a combination of single-cell analysis with cDNA microarrays is a highly desirable paradigm whereby expression profiles of single populations of neuronal and noneuronal subtypes can be analyzed and compared under normal and pathological conditions. The pattern of mRNA expression in a subpopulation of single neurons may be more informative

than patterns derived from whole brain or regional tissue homogenates, as each neuronal subtype is likely to have a unique molecular signature in normal and pathological states. Moreover, analysis of single neurons avoids potential overlap and/or contamination of expression profiles from noneuronal cells, epithelial cells, and vascular elements. Another goal is to combine these state-of-the-art expression profiling techniques with coordinated biochemical and morphological examinations within the same brains. Clearly, certain biochemical studies cannot be performed on fixed tissues, but can be applied to littermates in the case of animal models and on (unfixed) opposite hemispheres of human brains when available. Moreover, quantitative and qualitative morphological examinations are possible in the actual tissue sections used for microaspiration and subsequent cDNA array analysis. The emergence of high throughput proteomics also raises the distinct possibility of coordinated genomic and proteomic profiling within the same experimental paradigm.

In conclusion, simultaneous comparison of multiple genes from relevant classes of transcripts will help to elucidate molecular mechanisms underlying the pathogenesis of neurodegenerative and neuropsychiatric disorders. Furthermore, single-cell mRNA analysis has the potential for development of novel pharmacotherapeutic agents that target vulnerable gene(s) and gene products within specific cell types.

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