Single Cell Gene Expression Profiling in Alzheimer's Disease

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Summary: Development and implementation of microarray techniques to quantify expression levels of dozens to hundreds to thousands of transcripts simultaneously within select tissue samples from normal control subjects and neurodegenerative diseased brains has enabled scientists to create molecular fingerprints of vulnerable neuronal populations in Alzheimer's disease (AD) and related disorders. A goal is to sample gene expression from homogeneous cell types within a defined region without potential contamination by expression profiles of adjacent neuronal subpopulations and nonneuronal cells. The precise resolution afforded by single cell and population cell RNA analysis in combination with microarrays and real-time quantitative polymerase chain reaction (qPCR)–based analyses allows for relative gene expression level comparisons across cell types under different experimental conditions and disease progression. 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 of neurodegeneration as well as postmortem human brain tissues. Gene expression analysis in postmortem AD brain regions including the hippocampal formation and neocortex reveals selectively vulnerable cell types share putative pathogenetic alterations in common classes of transcripts, for example, markers of glutamatergic neurotransmission, synaptic-related markers, protein phosphatases and kinases, and neurotrophins/neurotrophin receptors. Expression profiles of vulnerable regions and neurons may reveal important clues toward the understanding of the molecular pathogenesis of various neurological diseases and aid in identifying rational targets toward pharmacotherapeutic interventions for progressive, late-onset neurodegenerative disorders such as mild cognitive impairment (MCI) and AD. **Key Words:** Microarray, neurodegeneration, RNA amplification, aging, qPCR, mild cognitive impairment, molecular fingerprint, postmortem human brain.

INTRODUCTION

Azheimer's disease (AD) is the most common cause of dementia worldwide, and it affects approximately 5% of people over the age of 60 and 50% of people over the age of 85.[1,2] Azheimer's disease is characterized clinically by progressive cognitive decline, loss of executive function, and memory deficits eventually leading to incapacitating dementia before death, and pathologically by the deposition of filamentous material in intracellular and extracellular compartments in the form of neurofibrillary tangles (NFTs), neuropil threads (NTs), and senile plaques (SPs), predominantly within the hippocampal formation and neocortex.[3–6] Although there is widespread decline in various neurotransmitter systems as AD progresses, most consistent deficits and cell loss are observed in long projection neurons, including cholinergic basal forebrain (CBF) neurons of the nucleus basalis (NB),[7–10] glutamatergic neurons within the entorhinal cortex and neocortex,[11,12] and glutamatergic layer II/III and V corticocortical projection neurons of secondary association cortex,[13] leading to regionally specific synaptic and connectivity-based degeneration. A diagnosis of AD is made postmortem on aged patients who display progressive cognitive decline, neuron loss, and the presence of abundant SPs and NFTs.[14,15] Although dementia associated with unsuccessful aging has been documented for centuries,[16] it was Dr. A. Alzheimer[17] who first described the clinicopathological features of the disease that today bears his name. Recent advances in genetics, cell biology, and molecular neuroscience have provided novel approaches to evaluate the neurodegenerative processes underlying the pathophysiology of AD.

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ALZHEIMER'S DISEASE GENETICS: AMYLOID-β PROTEIN PRECURSOR AND PRESENILIN

Genetic studies of the familial forms of Alzheimer's disease (FAD) along with the more prevalent sporadic form of AD have demonstrated that the entity designated as AD is a heterogeneous disorder with differing etiologies that share similar clinical and pathological phenotypes.[3,18] Notably, SP deposition is a potentially pathological process whereby amyloid fibrils of approximately 5–10 nm in diameter accumulate within extracellular compartments of the CNS, vasculature, and peripheral tissues in AD.[4,19] Amyloid fibrils are highly insoluble and display Congo red birefringence or thioflavine-S (TS) fluorescence due to a preferred β-pleated sheet conformation. The extracellular accumulation of abundant SPs within the hippocampal formation and neocortex of elderly people is a necessary prerequisite for the postmortem diagnosis of AD.[14,15] Several proteins are associated with SPs in AD brain, including the nonamyloid component of plaques (NACP), a 35-amino-acid proteolytic fragment of the α-synuclein protein found in Lewy bodies,[20,21] α1-anti-chymotrypsin, chromogranin A, serum amyloid P, heparan sulfate proteoglycan, several complement proteins (e.g., Clq, C3c, and C5b-9), and apolipoprotein E (APOE).[22–24] However, the major protein species purified from extracellular plaques (SPs and amyloid surrounding cerebral vasculature) are the ~4 kDa amyloid-β peptide (Aβ) peptides.[25,26] Although SPs are a distinguishing pathological hallmark of AD, heterogeneity exists in their morphology and composition. Senile plaques are categorized as neuritic plaques or diffuse plaques. Neuritic plaques have a dense Aβ-containing core and fibrillar corona, as well as an abundance of hyperphosphorylated tau-containing dystrophic neurites dispersed throughout the corona and core regions. Neuritic plaques are associated with inflammatory activation as microglial cells permeate the cytological boundaries of the fibrillar corona and reactive astrocytes are observed around the periphery.[27–31] Neuritic plaques are found within the AD brain and are also observed readily by Congo red birefringence or TS staining. Diffuse plaques, as their name suggests, lack a definitive amyloid core, contain few or no dystrophic neurites, do not produce an inflammatory response, nor are they visualized as readily as neuritic plagues by Congo red or TS staining. Diffuse plaques are identified optimally via immunocytochemistry using antibodies directed against Aβ peptides.[32,33] Presently, little or no correlation has been found between the quantity of extracellular SPs and/or Aβ burden and measures of cognitive/neurological dysfunction.[34–36] In contrast, a consensus is beginning to emerge that implicates nonfibrillar intracellular Aβ oligomers consisting principally of Aβ(1–42) as toxic intermediates that cause biochemical and neuropathological alterations in the AD brain and mouse models of cerebral amyloidosis.[37–40]

In addition to AD, several other neurological disorders involve cerebral amyloid deposition. For example, Down's syndrome (DS or Ts21) patients invariably develop AD pathology by their fourth decade of life and SPs have been identified in Ts21 patients by early adolescence.[41,42] The coincidence of AD pathology in Ts21 and the localization of the amyloid-β protein precursor (APP) gene to chromosome 21 was the initial impetus for genetic analyses of FAD on the chromosome 21 locus. Presently, 29 mutations in the APP gene account for a minor percentage of FAD kindreds (4 deemed to be nonpathogenic; Alzheimer Disease Mutation Database http://www.molgen.ua.ac.be/ADMutations), but provide genetic evidence for the abnormal function of APP processing and Aβ deposition in the pathogenesis of AD.[43–45] Along these lines, mice overexpressing FAD-related APP transgenes display increased Aβ peptide (especially Aβ(1–42) production and plaque deposition.[46–48]

Autosomal dominant mutations in genes encoding presenilin 1 (PSEN1) on chromosome 14 and presenilin 2 (PSEN2) on chromosome 1 account for the majority of FAD kindreds identified to date.[49–51] PSEN1 and PSEN2 are transmembrane proteins of approximately 50 kDa that share 67% sequence homology and are localized primarily to the endoplasmic reticulum and Golgi apparatus by immunocytochemistry.[50–52] PSEN1 and PSEN2 mRNA and protein are distributed throughout mouse, rat, monkey, and human tissues, including the brain, with notable enrichment in the hippocampus, entorhinal cortex, and cerebellum.[52,53] PSEN1 is hypothesized to be an integral component of the γ-secretase complex (along with nicastrin, pen-2, and aph-1, among others) that mediates the intramembrane proteolysis of APP to generate Aβ peptides and carboxy-terminal fragments of APP (CTFs).[54,55] Cell culture and transgenic mouse studies have also detected an interaction between mutations in the PSEN1 and PSEN2 genes observed in FAD kindreds and increased production of the amyloidogenic Aβ fragment [Aβ(1–42)].[46,56,57] Specifically, an increased ratio of Aβ(1–42)/Aβ(1–40) caused by defective presenilins may act as a seed or nidus for fibrillar Aβ deposition and intraneuronal accumulation, as Aβ(1–42) rapidly forms fibrils *in vitro*, is the principal component of SP lesions, and has been observed within intracellular stores.[38,58–60] Presently, 158 mutations (3 nonpathogenic) have been identified within the PSEN1 gene, and 11 (1 nonpathogenic) mutations have been detected in the PSEN2 gene (Alzheimer Disease Mutation Database).

Not all known FAD kindreds have been linked to PSEN1, PSEN2, or APP mutations, therefore other mutations linked to FAD await identification. A successful

method to delineate potential causal/susceptibility genes is linkage analysis using a genome-scan approach. This strategy employs microsatellite markers comprised of polymorphisms that likely represent random genetic variations.[61] Microsatellite maps have been generated that span the entire genome,[62] and research groups have used linkage-based approaches to find candidate genes associated with several progressive neuropathological disorders, including possible candidates for late-onset AD on chromosomes 10, 12, and 20.[63–65] In addition, a multitude of mouse models of APP and PSEN1 (as well as transgenic mice expressing both mutant APP and PSEN1) overexpression have been generated[40,46,48,56,66] that provide insight into potential mechanisms by which these proteins that are mutated in the FAD kindred may play a role in the pathophysiology of AD.

TAU MUTATIONS AND NEURODEGENERATIVE DISORDERS

Tau is a low-molecular-weight microtubule–associated protein found in the CNS and peripheral nervous system and is localized primarily to axonal compartments in normal brain.[67,68] Abnormally phosphorylated tau is observed within neurofibrillary lesions (NFTs, NTs, and dystrophic neurites) of the AD brain and other tangle-bearing disorders including Pick's disease, progressive supranuclear palsy, and corticobasal degeneration, among others.[3,69] Cloning of the tau gene revealed that multiple tau isoforms are produced from a single gene on chromosome 17 through alternative mRNA splicing.[70,71] Six isoforms of tau protein are found in the adult human brain: three isoforms with three tandem microtubule binding repeats (3Rtau) and three isoforms with four tandem microtubule binding repeats (4Rtau) of 31 or 32 amino acids in the carboxy-terminus of the molecule, and these tandem repeat regions contain microtubule binding domains.[70–72] Tau isoforms also differ by 29 or 58 amino acid inserts (0, 1, or 2 inserts; FIG. 1) in the amino-terminal region of the molecule.[70–72] Tau promotes the polymerization of tubulin monomers into microtubules.[72–74] Phosphorylation negatively regulates tau binding to microtubules.[74] Phosphorylation sites on the tau molecule occur at many proline-directed serine/threonine sites, although there are reports of phosphorylation at nonproline-directed sites.[71,75,76] Mutations in the human tau gene were discovered in a group of frontotemporal dementias (FTDs) termed chromosome 17-linked frontotemporal dementia and parkinsonism (FTDP-17).[69,77–79] At present, 40 different mutations (18 nonpathogenic) within the tau gene that cause FTD have been identified (Alzheimer Disease Mutation Database), including missense mutations in exons 9, 10, 12, and 13, as well as point mutations in the 5′ splice donor site of exon 10.[69,80,81] Many of these mutations result in the

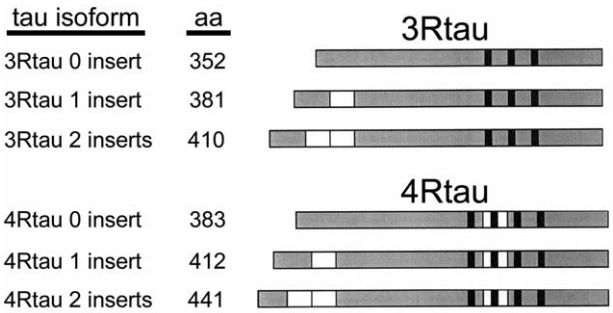


FIG. 1. Schematic diagram illustrating the six tau isoforms present in human brain. The number of amino terminal inserts (0, 1, or 2 inserts; white boxes) is depicted along with the predicted amino acids (aa) for each isoform. Tau isoforms are presented in terms of microtubule binding repeats (3Rtau, black boxes; 4Rtau, black and white boxes illustrate the additional microtubule binding repeat of 4Rtau). This schematic illustration is adapted from two of our previously published reports. [3,73]

intraneuronal accumulation of hyperphosphorylated tau and NFTs. Neuronal cell loss is also observed, although cell death does not always occur through an NFT-bearing mechanism.[82,83] FTDP-17 kindreds display a wide phenotypic variety that may reflect the function (i.e., dysfunction and/or toxic gain of function) of individual tau mutations, as mutations in exons 9, 10, and 12 lie within the microtubule binding domains of tau, whereas mutations in exon 13 affect the carboxy-terminus of the microtubule binding region.[78,82,84] Further understanding of the genetics of tauopathies may occur from investigations of genetically engineered animals including the htau,[85,86] P301L,[87,88] and R406W[89] mouse models.

APOLIPOPROTEIN E AS A RISK FACTOR FOR AD

In addition to the APP, PSEN1, and PSEN2 mutations found in FAD and the tau mutations found in FTD, the risk of developing sporadic AD (>95% of affected patients are sporadic cases to date) has been linked to the APOE gene on chromosome 19.[90,91] Three major alleles have been identified, APOE ε2, APOE ε3, and APOEε4, with the APOE ε3 allele being the most common. Individuals homozygous for the APOE ε4 allele are more likely to develop sporadic AD or FAD at an earlier age of onset compared with individuals with one (heterozygous) or no copies of the APOE ε4 allele.[92–94] In contrast, the presence of the APOE ε2 allele may decrease the risk for AD but is associated with a higher risk of hemorrhage due to cerebral amyloid angiopathy.[95] APOE is a 34-kDa glycoprotein that is synthesized principally in the liver and functions in lipid transport, notably cholesterol, and metabolic pathways.[96] The brain is also enriched in APOE mRNA and protein, with astrocytes being the primary cell type for protein localization, particularly in the aged brain,[91,97] although APOE is present in neurons within the human brain.[98] APOE

binds to soluble forms of Aβ peptides *in vitro* and isoform-specific interactions between APOE and amyloid fibril formation have been demonstrated.[99–101] APOE also interacts with tau in its nonphosphorylated state *in vitro* in an isoform-specific manner, with the APOE ε3 allele binding with a higher affinity to tau than the APOE ε4 allele.[102] APOE immunoreactivity has also been localized to SPs and NFTs within the AD brain and other neurodegenerative disorders.[98,103,104] However, the function of APOE in the normal brain as well as its role in the pathogenesis of AD is presently unknown. One hypothesis is that APOE, notably the ε4 allele, may act as a pathological chaperone to increase the deposition, or block the reuptake, of soluble Aβ peptides and/or hyperphosphorylated tau.

REGIONAL ASSESSMENTS OF GENE EXPRESSION IN THE AD BRAIN

Newly developed molecular and cellular approaches to neuroscience have enabled the initiation of high-throughput analysis of AD pathophysiology. To evaluate molecular events associated with the mechanisms of AD pathogenesis in animal models and human postmortem tissues, microarray studies and other downstream genetic analyses are performed at the regional and cellular levels to characterize transcriptional patterns, or mosaics, that may provide clues into some of the mechanism(s) underlying pathological events. An overriding goal is to apply functional genomics and proteomics-based approaches to neurodegenerative disease research to develop useful biomarkers for eventual pharmacotherapeutic development and disease prevention. At present, the molecular and cellular basis of AD pathogenesis is not well understood. Regional analysis of gene expression is a widely used paradigm because of the relatively large amounts of RNA that can be extracted from carefully dissected frozen animal model tissues as well as postmortem human brain tissues, as evidenced by reports on amyloid overexpression[105,106] and several neurodegenerative disorders, including AD.[107–116] An advantage of regional gene expression analysis is that, in most cases, extracted RNA is sufficient to generate significant hybridization signal intensity for microarray analysis, enabling the analysis of thousands of targets without necessitating RNA amplification protocols. However, expression profiles garnered from regional dissections cannot discern molecular signatures in discrete neuronal populations nor can regional assessments evaluate differences in admixed neuronal and nonneuronal populations. Relatively new RNA amplification procedures have been implemented to analyze gene expression profiles of single neurons and homogeneous neuronal populations.[117,118] A decided advantage of single cell and single population gene profiling is that different cell types can be identified and microaspirated based upon their signature neurochemical phenotype. For example, CBF neurons[73,119,120] and midbrain dopaminergic neurons[121–123] can be demarcated by selective expression of phenotypic markers and isolated for microdissection, RNA amplification, and subsequent microarray analysis. Additionally, cells that lack a distinct and/or selective phenotype can be analyzed using a variety of histochemical stains, including cresyl violet, hematoxylin and eosin, and thionin[124–127] for downstream genetic applications. Discrimination of adjacent and admixed cell types enables the differentiation of neuronal subtypes as well as neurons from glia, vascular epithelia, and other nonneuronal cell types within the brain. This type of functional genomics approach is ideal for experiments designed to evaluate potential molecular pathogenetic mechanisms underlying the selective vulnerability of specific neuronal populations affected in AD and other neurodegenerative disorders.

ACQUISITION OF CELL POPULATIONS FOR FUNCTIONAL GENOMICS

Alzheimer's disease is hypothesized to present clinically once an undetermined threshold number of critical hippocampal and neocortical neurons lose the ability to perform normative functions and subsequently fail to respond to fluctuations in the external and internal milieu. Degenerating neurons become progressively disconnected from their afferent and efferent projection sites, eventually succumbing to frank neurodegeneration. The brain is a complex structure with heterogeneous neuronal (e.g., pyramidal neurons and interneurons) and nonneuronal cell populations (e.g., glial cells, epithelial cells, inflammatory cells, and vascular elements). Thus, developing a set of molecular and cellular criteria that differentiates vulnerable neurons from those that are not affected during the course of AD is of tantamount importance. A key factor that determines specificity of sophisticated genetic testing methods is the purity and precise acquisition of identified cells for subsequent microarray and qPCR analysis. Accordingly, single cell and population cell molecular fingerprinting necessitates accurate, nondestructive isolation of cells from optimally prepared tissue sections.[118,128,129] Two popular and effective microdissection methodologies include laser capture microdissection (LCM) and microaspiration. Laser capture microdissection employs a high-energy laser source that separates desired cells from the remainder of a tissue section and facilitates transfer of the identified cells to a microfuge tube for downstream genetic analysis.[130,131] Single cells as well as hundreds of cells and other elements can be acquired quickly via LCM. Individual cells and pooled populations of cells can be visualized by immunocytochemical and/or histochemical

procedures for optimal identification of specific cells of interest for subsequent downstream genetic analyses, including microarray studies and qPCR in animal models of neurodegenerative disorders and postmortem human AD brain tissues.[124,125,132,133] Microaspiration is another technique used to isolate individual neurons and populations of homogeneous cells. This procedure 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 something using a microelectrode and excised. Microaspiration results in accurate dissection of the neurons of interest with minimal disruption of the surrounding neuropil.[134–138] 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, including analysis of postmortem AD tissues.[73,119,120,134,135,138] Microaspiration provides a more accurate dissection of single cells than LCM but is more labor intensive and has a significantly lower throughput potential.[118]

RNA AMPLIFICATION STRATEGIES

RNA amplification procedures are now used routinely to amplify input RNA to increase signal detection for microarray analysis and other downstream genetic analyses. RNA amplification methods preserve the original quantitative relationship(s) in an amplified gene population, facilitating downstream quantitative analysis. Amplified antisense RNA (aRNA) amplification, developed by Eberwine and colleagues,[139–141] utilizes a T7 RNA polymerase-based amplification procedure that allows for quantitation of the relative abundance of gene expression levels. aRNA products maintain a proportional representation of the size and complexity of input mRNAs.[139,142] aRNA synthesis amplifies genetic signals from limited amounts of fresh, frozen, or fixed tissues and cells and has been used successfully to analyze molecular fingerprints from AD and control neurons, including mRNAs accessed from SPs and NFT-bearing neurons.[134,135,137,143,144] Although aRNA is a complicated series of procedures, successful results have been generated with microaspirated animal model and postmortem human brain tissue samples using a wide variety of microarray platforms and several kits that use aRNA technology to amplify small amounts of RNA are available commercially.

A novel technology for RNA-based single-primer isothermal amplification (Ribo-SPIA) yielding high-fidelity RNA amplification for gene expression analysis has been developed and marketed by NuGEN (San Carlos, CA) and is of interest to molecular neuroscientists performing microarray evaluations.[145,146] Ribo-SPIA isothermal amplification technology is different from methods that use *in vitro* transcription with a bacteriophage transcription promoter such as T7 polymerase. Ribo-SPIA is a linear RNA amplification procedure that entails the formation of a double-stranded cDNA as a substrate for subsequent single-primer isothermal amplification, effectively generating multiple copies of single-stranded DNA products that are complementary to the initial input mRNA source.[147,148] A single Ribo-SPIA amplification can generate amplified RNA for multiple hybridization experiments from less than ~5 ng of total RNA.[147]

A new methodology has been developed in our laboratory to amplify RNA from minute amounts of starting material.[117,149,150] This method, termed terminal continuation (TC) RNA amplification, enables an efficient means of second-strand cDNA synthesis using a two-primer system. An RNA synthesis promoter is attached to the 3′ and/or 5′ region of cDNA using the TC mechanism.[149,150] The orientation of amplified RNAs is "antisense," or a novel "sense" orientation, and one round of TC RNA amplification is sufficient for downstream genetic analyses.[117,126,149,150] Terminal continuation RNA amplification is used for many downstream applications, including gene expression profiling, 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. Regional and single cell gene expression studies within the brains of animal models and human postmortem AD, mild cognitive impairment (MCI), and normal control brain tissues have been performed via microarray and qPCR analysis coupled with TC RNA amplification.[73,119,120,128,129,132,133,138]

MICROARRAY PLATFORMS

Methodological and technical advances have enabled the development of myriad microarray platforms allowing for high-throughput analysis of dozens to hundreds to thousands of genes simultaneously. Microarrays represent miniaturized, high-density dot-blots that take advantage of complementary hybridization between nucleic acids.[151–153] Synthesis of cDNA microarrays entails adhering cDNAs or expressed sequence–tagged cDNAs (ESTs) to solid supports such as glass slides, plastic slides, or nylon membranes. A parallel technology uses photolithography to adhere oligonucleotides to array media.[154] The anchored cDNA/EST/oligonucleotide is commonly called the microarray target. Target length varies from short oligonucleotides to chromosomal fragments, depending on the array platform design. Sample RNA is used to generate labeled probes via biotinylated, fluorescent, or radioactive label incorporation. Arrays are washed to remove nonspecific background hybridization

and imaged using a laser scanner for biotinylated/fluorescently labeled probes and a phosphor imager for radioactively labeled probes. A target-labeled probe complex emits a quantifiable signal that is proportional to the abundance of the labeled probe in the sample. The specific signal intensity (minus background) of total (or amplified) RNA bound to each probe set (e.g., oligonucleotides or cDNAs/ESTs) is expressed as a ratio of the total hybridization signal intensity, reducing variation across array platforms due to differences in specific activity of the probe and absolute quantity of probe present. Quantification of hybridization signal intensity is performed to evaluate relative expression levels of each cDNA, EST, or oligonucleotide located on the array platform. Gene expression is then assessed using statistical 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.[155,156] A prominent caveat of microarray array analysis is that coordinate changes in proteins encoded by the genes of interest are common, but not absolute.[157]

EXPRESSION PROFILE ANALYSIS OF SENILE PLAQUES IN THE AD HIPPOCAMPUS

Although extensive analysis of the immunocytochemical makeup of SPs has been performed, few data exist on the nonproteinaceous components of these lesions. The presence of RNA species (e.g., mRNA, rRNA, and tRNA) in SPs was evaluated using acridine orange (AO) histofluorescence, alone or in combination with TS staining and immunocytochemistry, in AD brains and related neurodegenerative disorders that display abundant SPs.[31,158] AO is a fluorescent dye that intercalates selectively into nucleic acids and has been used to detect RNA and DNA in brain tissues.[31,127,138,159] Acridine orange can be employed in combination with immunocytochemistry to identify cytoplasmic RNAs and specific antigens of interest (FIG. 2A) and is compatible with confocal microscopy.[158,160] Quantitative analysis of double-labeled sections demonstrates approximately 55% of TS-stained SPs also contain AO labeling,[31,158] indicating the presence of RNA species in a significant population of SPs. The observed sequestration of RNA SPs in AD and related disorders prompted single cell analysis of SPs in sections of AD hippocampus. The expression profile of SPs was compared with individual CA1 neurons and surrounding neuropil of control brains using single cell RNA amplification coupled with custom-designed cDNA arrays.[134] Results indicate that SPs harbor two distinct populations of gene expression levels. One cluster of transcripts contains high-abundance genes including APP, tau, Bcl-2, bax, protein phosphatase subunits, and several ionotropic glutamate receptors (GluRs).[128,134] A second grouping of transcripts contains low-abundance genes including neurofilament subunits and glial-enriched mRNAs [e.g., GFAP, interleukin-1 (IL-1), and the receptor for advanced glycation end-products (AGER)].[128,134] A molecular fingerprint of SPs obtained from the CA1-subiculum region of AD brains is presented relative to CA1 neurons in normal brains (FIG. 2D). Additionally, a representation of glial-associated mRNAs enriched in neuropil microaspirated from AD brains is presented relative to SPs in Figure 2. The presence of mRNA species in extracellular SPs is validated further by combined TS and *in situ* hybridization histochemistry using a probe against cyclic AMP response element binding protein (CREB) in addition to PCR to identify APP isoforms from individual SPs.[134] Combined single cell expression profiling, *in situ* hybridization, and PCR datasets indicate that multiple mRNA species are found in individual, extracellular SPs of the AD hippocampus. Furthermore, expression profiles amplified from SPs are predominantly neuronal. These observations suggest that SPs sequester the remnants of degenerating neurons and their processes. Microglial cells have been identified within SPs and astrocytosis occurs around SPs (FIG. 2),[29,31,161] but relatively low levels of glial-derived mRNAs are found in SPs, likely reflecting a lower abundance of RNAs in glial cells,[162] although expression profiling of astrocytes has been performed successfully in animal models.[163,164]

EXPRESSION PROFILE ANALYSIS OF NEUROFIBRILLARY TANGLES IN AD HIPPOCAMPAL CA1 NEURONS

The pathogenesis of NFTs in AD and related disorders is not well understood and the molecular pathophysiology of these lesions is beginning to be evaluated in animal models of tauopathy and in postmortem human brains.[85,86,128,129,135] For example, quantitative analysis of hippocampal tissue sections double labeled for AO and TS demonstrates approximately 80% of TS-positive NFTs are AO-positive.[31,158] We have employed a single cell microarray analysis scheme to identify transcripts that are differentially regulated in CA1 neurons that bear NFTs in AD relative to non-tangle–bearing CA1 neurons in normal-aged control brains. The underlying hypothesis of this study is that alterations in the expression of specific transcripts will reflect mechanisms underlying the formation of NFTs and their biological consequences in affected neurons. Single cell RNA amplification has been performed in combination with high-density and custom-designed array platforms. Relative to normal CA1 neurons, NFT-bearing neurons in AD displayed significant reductions in several classes of mRNAs implicated in the neuropathology of this disorder, including

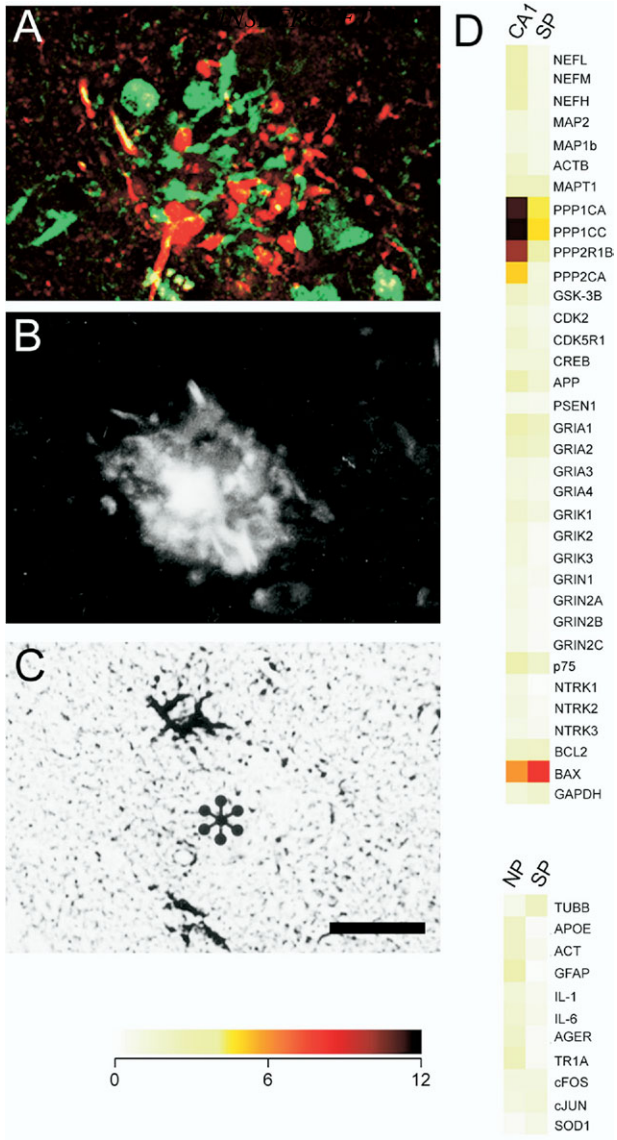


FIG. 2. Expression profiling within SPs obtained from AD hippocampus. (A) Confocal laser scanning microscopy of an SP labeled by AO histofluorescence and immunocytochemistry against hyperphosphorylated tau. AO is pseudocolored green, while the immunocytochemical labeling is pseudocolored red and the colocalization of the two signals is represented as orange–yellow. Although abundant AO labeling is present within dystrophic processes of this SP from an AD brain, only occasional colocalization is observed between the AO signal and PHF1-immunoreactive dystrophic neurites, indicating that the AO signal is likely originating from amyloid fibrils. (B) Double-label preparations indicating the association between astrocytes and AO-labeled SPs within the entorhinal cortex of an AD patient. B depicts an AO-labeled entorhinal cortex SP. (C) Bright-field photomicrograph of the same section as B, demonstrating the apposition, but not colocalization, of GFAP-immunoreactive astrocytes and AO-labeled SP (asterisk). Scale bar A–C: 25 μm. (D) Dendrogram illustrating expression profiles garnered from CA1 pyramidal neurons using normal control brains and SPs from AD brains. In addition, a series of glial-associated mRNAs are presented to 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. Note the use of the NCBI-Unigene annotation for Figs. 2D and 3F: 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, BCL2-associated X protein; BCL2, B-cell lymphoma 2; CAMK2, calcium/calmodulin-dependent protein kinase II, alpha; CDK2, cyclin-dependent kinase 2; CDK5, cyclin-dependent kinase 5; CDK5R1, cyclin-dependent kinase 5, regulatory subunit 1 (p35); DAT, dopamine transporter; DRD1, dopamine receptor 1; DRD2, dopamine receptor 2; DRD3, dopamine receptor 3; DRD4, dopamine receptor 4; DRD5, dopamine receptor 5; cFOS, cellular oncogene fos; cJUN, jun oncogene; CREB, cAMP responsive element binding protein; CTSD, cathepsin D; FAK, focal adhesion kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GLRX, glutaredoxin; GRIA1, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid 1 receptor (AMPA1); GRIA2, AMPA2; GRIA3, AMPA3; GRIA4, AMPA4; GRIK1, kainate receptor 1; GRIK2, kainate 2; GRIK3, kainate 3; GRIN1, N-methyl D-aspartate receptor 1 (NMDAR1); GRIN2A, NMDAR2A; GRIN2B, NMDAR2B; GRIN2C, NMDAR2C; GSK-3B, glycogen synthase kinase-3 beta; HSPG, heparan sulfate proteoglycan; IL-1, interleukin 1; IL-6, interleukin 6; MAP1b; microtubule-associated protein 1b; MAP2, microtubule-associated protein 2; MAPK1, mitogen-activated protein kinase 1 (p44); MAPK2, mitogen-activated protein kinase 2 (p42); MAPT1, three-repeat tau; MAPT2, four-repeat tau; NEFH, neurofilament heavy subunit; NEFL, neurofilament light subunit; NEFM, neurofilament medium subunit; NTRK1, high affinity nerve growth factor receptor trkA; NTRK2, trkB; NTRK3, trkC; p75, low affinity nerve growth factor receptor; PPP1CA, protein phosphatase 1, catalytic subunit, alpha isoform; PPP1CC, protein phosphatase 1, catalytic subunit, gamma isoform; PPP2R1B, protein phosphatase 2, regulatory subunit A, beta isoform; PPP2CA, protein phosphatase 2, catalytic subunit, alpha isoform; PPP2CG, protein phosphatase 2, catalytic subunit, gamma isoform; PSEN1, presenilin 1; PSEN2, presenilin 2; SNCA, alpha-synuclein; SNCB, beta-synuclein; SOD1, superoxide dismutase 1; SYN1, synapsin I; SYP, synaptophysin; SYT1, synaptotagmin I; TR1A, tumor necrosis factor receptor superfamily member 1A; TUBB, beta-tubulin.

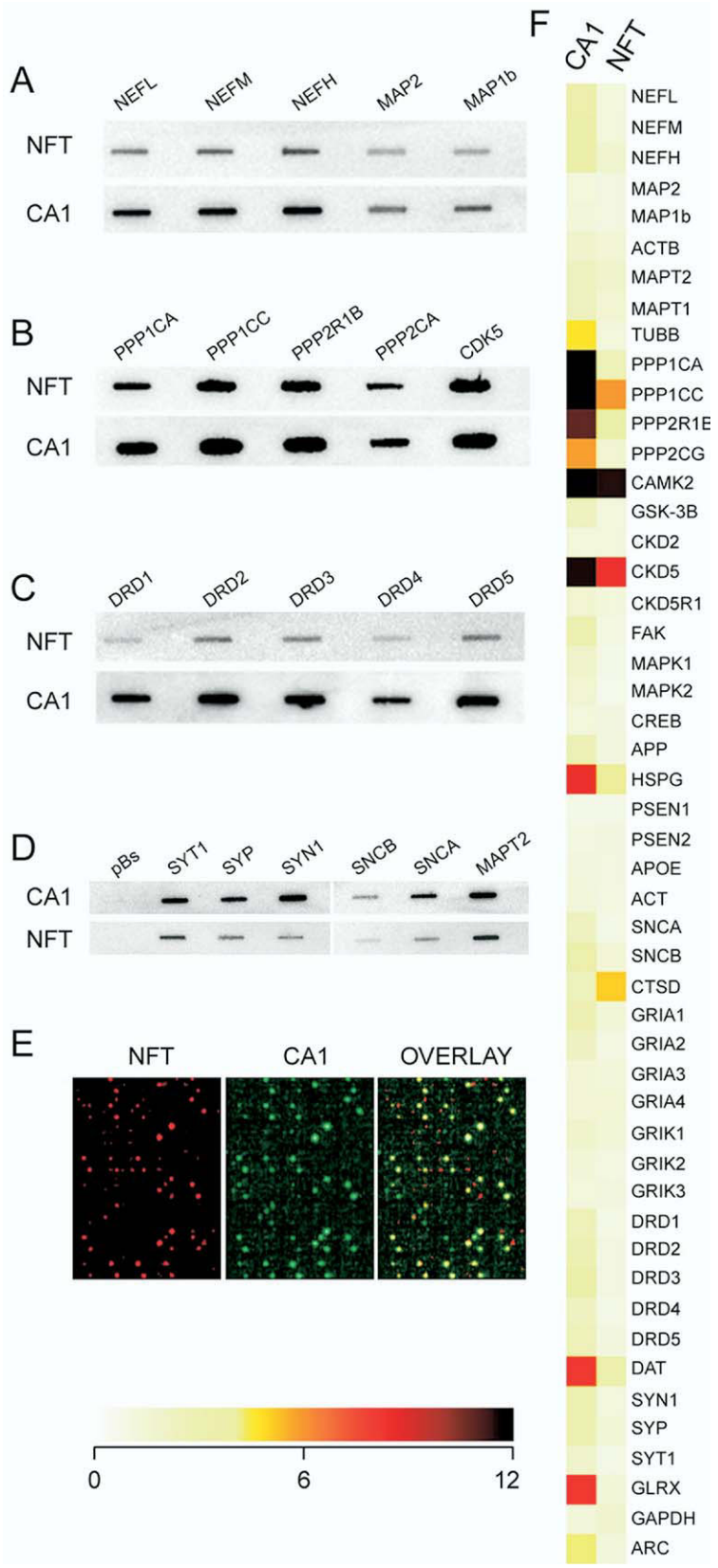
cytoskeletal elements, dopamine receptors, GluRs, protein phosphatases and kinases, and synaptic-related markers (FIG. 3).[118,135] Total hybridization signal intensity on the array platform is down-regulated in single AD NFT-bearing neurons compared with normal CA1 neurons by approximately 30%, consistent with studies of total polyadenylated mRNA expression in AD.[165,166] A correspondence of approximately 85% between the two array platforms was 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.[135]

As a class of transcripts, mRNAs for protein phosphatase 1 and protein phosphatase 2 subunits displayed significant down-regulation in CA1 neurons with NFTs compared with those without NFTs (FIG. 3B), consistent with related studies implicating reduced protein phosphatase expression and activity as a potential contributing mechanism toward NFT formation.[167,168] For example, down-regulation of multiple ESTs linked to a single protein phosphatase subunit transcript was observed, including all five ESTs linked to the protein phosphatase 2, regulatory subunit A, beta isoform (PPP2R1B) on the high-density microarray. These observations are consistent with a similar level of relative down-regulation observed on the custom-designed cDNA array platform.[128,135] Tau hyperphosphorylation in AD and other tauopathies are likely due to dysfunction in protein phosphorylation and dephosphorylation via decreases in protein phosphatase abundance and/or activity, as well as concomitant increases in tau kinases.[18,167,169] Thus, developing pharmacotherapeutic interventions that ameliorate abnormal tau hyperphosphorylation through protein phosphatase and/or kinase modulation may become a viable and rational therapy for the treatment of disorders with profuse neurofibrillary pathology, including AD and FTDs.

In terms of neurotransmitter systems effected in single neurons, a two- to fourfold decrease in the expression of the mRNAs for dopamine receptors DRD1–DRD5 (NCBI/Unigene annotation) and the dopamine transporter is observed in NFT-bearing neurons in AD versus non-NFT–bearing neurons in control brains (FIG. 3C).[135] These observations are consistent with results displaying decreased DRD2 receptor binding in the AD hippocampus.[170,171] Moreover, single cell results illustrate advantages of employing single cell dopamine receptor mRNA analyses since antibodies and ligand-based studies have not been able to discriminate unequivocally between these dopamine receptor subtypes. In addition, the effects of aging on DRD1–DRD5 mRNA expression levels in hippocampal CA1 pyramidal neurons and entorhinal cortex layer II stellate cells have been evaluated in a cohort of normal control postmortem human brains aged 19–95 years.[137] Results indicate a significant age-related decline for DRD1–DRD5 mRNAs in CA1 pyramidal neurons with percent decline per decade for each receptor subtype being 5.2, 5.0, 11.2, 4.7, and 5.0%, respectively.[137] Down-regulation of dopamine receptor subtypes appears to be relatively selective, as no decrement in other mRNAs, including the cytoskeletal elements β-actin (ACTB), 3Rtau, and 4Rtau, is observed in CA1 pyramidal neurons in the same subjects.[137] In contrast, no significant changes in dopamine receptor subtype expression are observed in stellate cells across the same cohort. Deficits in dopaminergic neurotransmission contribute to cognitive decline associated with normal aging in the hippocampus of senescent rats and humans[172–174] and may be relevant toward understanding aspects of the pathophysiology associated with attention and memory deficits in progressive, late-onset neurodegenerative disorders such as AD.

Synaptic loss in AD is considered one of the hallmarks of the disorder and reduction in gene expression levels of synaptic-related markers is observed consistently in NFT-bearing CA1 neurons, implicating NFTs in synaptic damage to affected neurons. Specifically, synaptic-related markers that are down-regulated include synaptophysin (SYP; 2.2-fold), synaptotagmin I (SYT1; 2.0-fold), synapsin I (SYN1; 2.5-fold), α-synuclein (SNCA; 2.5-fold), and β-synuclein (SNCB; 2.3-fold) (FIG. 3D).[128,135] These data are consistent with observations that SYP mRNA levels are decreased in the AD hippocampus by qPCR and *in situ* hybridization.[175,176] Moreover, selective down-regulation of synaptic-related proteins appears to be an early manifestation of AD, as loss of SYP protein correlates with cognitive decline.[177–180]

Quantitative assessment of high-density cDNA microarrays revealed several ESTs are up-regulated in individual NFT-bearing CA1 pyramidal neurons in AD brains compared with normal CA1 neurons in age-matched control brains, including the lysosomal hydrolase cathepsin D (CatD or CTSD-Unigene abbreviation).[128,135] CTSD has been demonstrated to be up-regulated in endosomal and lysosomal compartments in AD brain and may have profound pathological sequelae.[181–183] An approximate twofold up-regulation of CTSD expression is observed on a custom-designed array platform and hyperphosphorylated tau positive NFTs colocalize with increased CTSD immunoreactivity in the same CA1 neurons.[3,135] Up-regulation of the acid hydrolase CTSD is consistent with a growing body of literature indicating that activation of the endosomal–lysosomal–autophagic system is a pervasive, early alteration in AD.[182,184–186] These data illustrate an experimental strategy to employ both high-density and custom-designed microarrays and single cell RNA amplification to identify alterations in the expression levels of numer-


A
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CA1
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PPP1CC
PPP2R1B
PPP2CG
CAMK2
GSK-3B
CKD2
CKD5
CKD5R1
FAK
MAPK1
MAPK2
CREB
APP
HSPG
PSEN1
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GRIK3
DRD1
DRD2
DRD3
DRD4
DRD5
DAT
SYN1
SYP
SYT1
GLRX
GAPDH
ARC

FIG. 3. Expression profile analysis of select transcripts in NFT-bearing neurons obtained from AD in comparison with non-tangle-bearing neurons from control brains. (A) Down-regulation in the relative abundance of neurofilament gene expression (2- to 4-fold decrease) in single NFT-bearing neurons. No differences in MAP1b or MAP2 expression were detected. (B) Down-regulation of several protein phosphatase subunits in NFT-bearing neurons. A representative custom cDNA array demonstrates a significant decrease in the relative abundance of mRNAs encoding protein phosphatase 1 subunits (PPP1CA, PPP1CC) and protein phosphatase 2 subunits (PPP2R1B, PPP2CA). (C) Dopamine receptors DRD1–DRD5 are significantly decreased in AD NFT-bearing CA1 neurons compared with nontangle–bearing CA1 pyramidal neurons in control brains. (D) Expression profiling revealed down-regulation of select synaptic-related markers including SNCA, SNCB, SYP, SYN1, and SYT1. In contrast, no differential regulation of 4Rtau (0 insert; MAPT2) or empty vector (pBs) was observed. (E) A portion of a representative high-density array, illustrating a wide range of hybridization signal using probes generated from NFT-bearing neurons (right; pseudocolored red), normal CA1 neurons (middle; pseudocolored green), and an overlay of NFT-bearing and normal CA1 neuron hybridizations (left). In the left panel, yellow indicates similar hybridization intensities for NFT-bearing neurons and normal CA1 neurons, green indicates a down-regulation in NFT-bearing neurons relative to normal CA1 neurons, and red denotes an up-regulation of an EST within diseased versus normal CA1 neurons. (F) Color-coded dendrogram depicting expression profiles garnered from CA1 pyramidal neurons from normal control brains and NFT-bearing CA1 pyramidal neurons from AD brains.

ous transcripts in AD NFT-bearing neurons compared with normal control CA1 neurons.

SINGLE CELL ANALYSIS OF CBF NEURONS IN AD

Cholinergic basal forebrain neurons supply the majority of cholinergic fibers to the hippocampal formation and cerebral cortex, are a key component of memory and attention circuits in the brain,[187–189] are selectively vulnerable in AD[9,190] and CBF neuron degeneration correlates with disease duration and cognitive decline.[7,191,192] Currently, molecular and cellular mechanisms underlying CBF dysfunction remain unknown. Single cell TC RNA amplification and custom-designed cDNA array technology have been employed to evaluate expression levels of several functional classes of mRNAs found in CBF neurons from normal-aged and AD subjects.[6,119,120,138] Similar to observations in NFT-bearing CA1 neurons,[135] down-regulation of synaptic-related markers (e.g., SYP and SYT1) and protein phosphatase 1 and protein phosphatase 2 subunit mRNAs, along with up-regulation of CTSD mRNA, is observed in AD compared with normal control CBF neurons.[128,138]

Attempts to analyze molecular and cellular alterations in specific tau isoforms within vulnerable neuronal populations have been hindered by the lack of antemortem, clinically well-characterized cases. Moreover, select populations of neurons degenerate, whereas others remain intact during the progression of AD.[118,126,129,193,194] Cholinergic basal forebrain neurons of the NB display phenotypic alterations in cholinergic markers and contain tau pathology in subjects characterized as MCI.[138,195,196] Single cell expression profiling was performed to assess the expression levels of the six tau isoforms (MAPT1–MAPT6; FIG. 1) and several other cytoskeletal elements from individual CBF neurons microaspirated using postmortem tissue samples obtained from 34 subjects clinically characterized antemortem with no cognitive impairment (NCI), MCI, and AD. These cases were obtained from the Religious Orders Study, an ongoing longitudinal clinicopathological study of aging and dementia in Catholic nuns, priests, and brothers.[6,138,197,198] Results indicate that tau transcript expression and other cytoskeletal elements (including neurofilament subunits and tubulin, among others) levels did not differ significantly across groups. However, when the ratios of 3Rtau/4Rtau were calculated, a significant difference in the proportion of 3Rtau/4Rtau mRNA was found in MCI and AD relative to NCI. Specifically, a decrement in the expression ratio of 3Rtau relative to 4Rtau was observed in both MCI and AD CBF neurons in all tau transcripts (e.g., 3Rtau/4Rtau 0 amino terminal insert, 1 amino terminal insert, and 2 amino terminal inserts).[73] The shift is due to a decrease in 3Rtau as opposed to an increase in 4Rtau levels. In addition, this shift in the 3Rtau/4Rtau ratio was present in NB neurons from MCI and AD, suggesting that this dysregulation impacts neuronal function and marks a transition from normal cognition to prodromal AD.

Based on these results, a 3Rtau/4Rtau ratio for CA1 pyramidal single cell gene expression profiles from cognitively normal aged controls and end-stage AD cases was calculated. There were no differences in total 3Rtau or 4Rtau expression between microaspirated CA1 neurofilament-immunoreactive from normal aged control brains and hyperphosphorylated tau-immunoreactive NFTs from AD brains.[135] Similar to the CBF data, there was a shift in the 3Rtau/4Rtau ratio in AD CA1 pyramidal neurons. In contrast to AD, age-related differences within CA1 pyramidal neurons and entorhinal cortex layer II/III stellate cells obtained from a cohort of normal controls aged 19–92 years were not observed in overall tau expression levels or the 3Rtau/4Rtau ratio.[73,137] The functional significance of a shift in the 3Rtau/4Rtau ratio in vulnerable neurons remains unknown. Studies indicate that 3Rtau and 4Rtau may play different roles in select neurons[199,200] and could be dysregulated differentially during the pathogenesis of various neurodegenerative diseases. For example, protein expression level studies link alterations in 3Rtau expression to NFT formation in AD, whereas alterations in 4Rtau expression levels are linked to tauopathies such as progressive supranuclear palsy and corticobasal degeneration.[81,201–205] These data suggest a subtle, yet pervasive change in gene dosage of

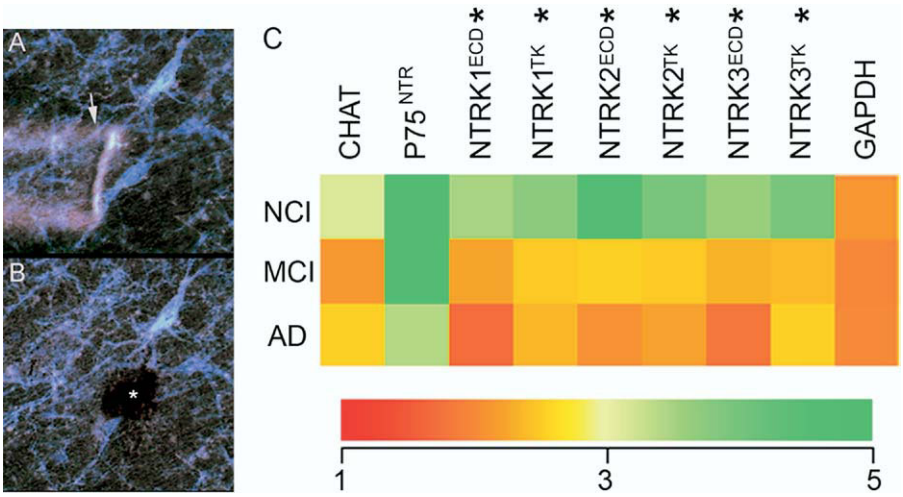

FIG. 4. Expression profiling of individual CBF neurons in NCI, MCI, and AD. (A) Dark-field photomicrograph of a representative $p75^{NTR}$–immunoreactive NB neuron before microaspiration. The white arrow points to the tip of a micropipette. (B) Same tissue section shown in A illustrating the site of the microaspirated neuron (white asterisk). (C) Expression profile analysis of $p75^{NTR}$, trkA (NTRK1), trkB (NTRK2), trkC (NTRK3), CHAT, and GAPDH derived from individual NB neurons from NCI, MCI, and AD subjects. Dendrogram with a color-coded scale illustrating relative expression levels. No significant differences are found for CHAT, $p75^{NTR}$, and GAPDH gene expression. In contrast, down-regulation (asterisk) of trkA, trkB, and trkC are observed in MCI and AD. ESTs identifying extracellular domain (ECD) and tyrosine kinase (TK) domains display down-regulation. The decrement of trk gene expression in MCI is intermediate relative to AD, indicating a step-down effect in expression levels from NCI to MCI to AD.

3Rtau and 4Rtau within vulnerable neurons in MCI and AD, which does not occur during normal aging. Shifts in the ratio of tau genes may be a fundamental mechanism whereby normal tau expression is dysregulated, leading to NFT formation.

Degeneration of the CBF system suggests that deficits in cortical cholinergic transmission mediated via NB neurons may contribute to the severe cognitive abnormalities seen in advanced AD.[9,206] Despite intense interest in the basal forebrain cholinergic cortical projection system, molecular and cellular mechanisms underlying NB neurodegeneration during the progression of AD remain unclear. Notably, CBF survival appears to require appropriate binding, internalization, and retrograde transport of the prototypic neurotrophin, NGF, which is synthesized and secreted by cells in the cortex and binds with its high-affinity (trkA) and low-affinity ($p75^{NTR}$) neurotrophin receptors produced within CBF neurons.[206,207] Since trkA and $p75^{NTR}$ protein levels are reduced within CBF neurons of people with MCI and mild AD, trkA and/or $p75^{NTR}$ gene expression deficits may drive NB degeneration.[6,196]

Using single cell expression profiling methods coupled with custom-designed cDNA arrays and validation with real-time qPCR and *in situ* hybridization, individual cholinergic NB neurons display a significant down-regulation of trkA, trkB, and trkC expression during the progression of AD.[120] Specifically, significant down-regulation of trkA, trkB, and trkC is observed in individual neurons microaspirated from AD and MCI compared with NCI.[120] An intermediate reduction is observed in MCI, with the greatest decrement in mild AD compared with controls. Moreover, down-regulation was found for two separate ESTs for each trk gene [e.g., ESTs targeted to the extracellular domain and tyrosine kinase domain].[120] In contrast, dysregulation of choline acetyltransferase and glyceraldehyde-3-phosphate dehydrogenase mRNA was not observed across clinical conditions (FIG. 4). Taken together, these findings indicate a relative selectivity in the alteration of high-affinity neurotrophin receptors within single NB neurons during prodromal stages of AD. Importantly, trkA, trkB, and trkC down-regulation is associated with cognitive decline as measured by a Global Cognitive Score (GCS) and the Mini-Mental State Examination (MMSE).[120] In contrast, there is a lack of regulation of $p75^{NTR}$ expression and no association between $p75^{NTR}$ expression and cognitive decline.[120,208] These observations were not facilitated by low GCS or MMSE scores observed in more severe AD cases examined in this study. Rather, these data indicate that virtually all of the MCI and AD cases display intermediate and lower expression levels, respectively, that contribute to the overall decrement in expression levels.[120] These results suggest that the onset of neurotrophic dysfunction in CBF neurons occurs during the earliest stages of cognitive decline and that deficits in trk expression are associated with the clinical presentation of the disease. Thus, trk defects may be a molecular marker for the transition from NCI to MCI and from MCI to frank AD.

CONCLUSION

Technical and methodological improvements in microaspiration techniques, RNA amplification, microarray analysis, and qPCR have led to an increase in the number

of studies of single cell gene expression. 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.[145,146,209,210] Unlike an organ that is comprised of one principal cell type, the brain contains a constellation of neuronal and nonneuronal populations of cells. Importantly, creating a molecular fingerprint of single neurons that are selectively vulnerable requires their precise localization within a defined brain region. Therefore, resolution at the level of homogeneous neuronal populations is optimal to create an expression profile for affected cells such as cholinergic NB neurons and CA1 pyramidal neurons. Simultaneous quantitative assessment of multiple transcripts by microaspiration, RNA amplification, and custom-designed cDNA microarray analysis provides a paradigm whereby the genetic signature of anatomically defined cells within a specific brain region can be differentiated from neighboring structures.[118,128,129,194] This experimental design allows for rigorous quantitative analyses of vulnerable cell types during the progression of clinical impairment. The unprecedented resolution afforded by single cell RNA analysis in combination with microarrays and qPCR-based analyses allows for relative gene expression level comparisons across cell types under different experimental conditions and disease states. A mosaic of gene expression levels 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 possible overlap and intermingling of expression profiles. 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, although high-throughput screening assays using single cells or populations of homogeneous cells *in vivo* are technologies that will require additional methodological and miniaturization development.

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