α7 Nicotinic Receptor Up-regulation in Cholinergic Basal Forebrain Neurons in Alzheimer Disease

Scott E. Counts, PhD; Bin He, MD; Shaoli Che, MD, PhD; Milos D. Ikonomovic, MD; Steven T. DeKosky, MD; Stephen D. Ginsberg, PhD; Elliott J. Mufson, PhD

Background: Dysfunction of basocortical cholinergic projection neurons of the nucleus basalis (NB) correlates with cognitive deficits in Alzheimer disease (AD). Nucleus basalis neurons receive cholinergic inputs and express nicotinic acetylcholine receptors (nAChRs) and muscarinic AChRs (mAChRs), which may regulate NB neuron activity in AD. Although alterations in these AChRs occur in the AD cortex, there is little information detailing whether defects in nAChR and mAChR gene expression occur in cholinergic NB neurons during disease progression.

Objective: To determine whether nAChR and mAChR gene expression is altered in cholinergic NB neurons during the progression of AD.

Design: Individual NB neurons from subjects diagnosed ante mortem as having no cognitive impairment (NCI), mild cognitive impairment (MCI), or mild to moderate AD were analyzed by single-cell AChR expression profiling via custom-designed microarrays.

Setting: Academic research.

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Results: Cholinergic NB neurons displayed a statistically significant up-regulation of α7 nAChR messenger RNA expression in subjects with mild to moderate AD compared with those with NCI and MCI (P < .001). No differences were found for other nAChR and mAChR subtypes across the cohort. Expression levels of α7 nAChRs were inversely associated with Global Cognitive Score and with Mini-Mental State Examination performance.

Conclusions: Up-regulation of α7 nAChRs may signal a compensatory response to maintain basocortical cholinergic activity during AD progression. Alternatively, putative competitive interactions of this receptor with β-amyloid may provide a pathogenic mechanism for NB dysfunction. Increasing NB α7 nAChR expression may serve as a marker for the progression of AD.

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Table. Case Demographics

<table>
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<tr>
<th>Variable</th>
<th>NCI (n=12)</th>
<th>MCI (n=10)</th>
<th>AD (n=12)</th>
<th>Total (N=34)</th>
<th>Group Comparison</th>
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<td>Age, y</td>
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<td>Male sex</td>
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<td>14 (41)</td>
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<td>18.8±2.3</td>
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<td>Mini-Mental State Examination score</td>
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<td>(NCI, MCI) &gt; AD</td>
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<td>Global Cognitive Score</td>
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<td>-0.9±0.5</td>
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<td>&lt;.001</td>
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<td>APOE ε4 allele</td>
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<td>6 (50)</td>
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</table>

Abbreviations: AD, mild to moderate Alzheimer disease; MCI, mild cognitive impairment; NCI, no cognitive impairment; PMI, postmortem interval.

Data are given as mean ± SD, number, or as percentages unless otherwise indicated.

b Bonferroni-type correction.

c Fisher exact test. Kruskal-Wallis test for all other values in this column.

CLINICAL AND PATHOLOGICAL EVALUATION

Details of the annual clinical and neuropsychological evaluations of the Rush Religious Orders Study cohort have been published.13 Cognitive testing was performed under the auspices of a neuropsychologist, and scores were available within the last year prior to death. A board-certified neurologist with expertise in the evaluation of older persons made a clinical diagnosis for each participant based on review of all clinical data and physical examination findings. Subjects were categorized as having NCI (n=12), MCI (n=10), or mild to moderate AD (n=12) (Table). The MCI population was defined as subjects who exhibited impaired neuropsychological test scores but did not meet the clinical criteria for AD recommended by the Joint Working Group of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s Disease and Related Disorders Association.12 These criteria are compatible with those used by experts in the field to describe subjects who are not cognitively normal but do not meet established criteria for dementia.18 None of the subjects examined reported a history of smoking or cholinesterase inhibitor therapy on entry into the cohort.

At autopsy, brains were removed from the calvarium and were cut into 0.5-cm-thick slabs using a transparent thermoplastic brain-cutting apparatus (produced by the University of Illinois, Chicago). The slabs were hemisected, and 1 hemispheric sphere was immersion fixed in a 4% paraformaldehyde solution in 0.1M phosphate buffer (pH 7.2), cryoprotected, and cut frozen into 40-µm-thick sections.13 Neuropathological assessments were performed by a neuropathologist blinded to clinical diagnosis. Cases were classified based on the National Institute on Aging and the Reagan Institute Working Group14 and Consortium to Establish a Registry for Alzheimer’s Disease criteria,15 as well as by Braak stage.16 Slabs from the opposite hemisphere were frozen at -80°C.

IMMUNOHISTOCHEMISTRY

Ribonuclease-free precautions were used throughout the experimental procedures. The presence of intact RNA in the tissue sections was confirmed by acidine orange histofluorescence and biochemical analysis (Agilent, Santa Clara, California).17,18 Tissue sections were processed using a monoclonal antibody raised against the human p75 neurotrophin receptor, which co-localizes with approximately 95% of all cholinergic basal forebrain neurons within the human NB.19 Tissue sections were incubated for 1 hour in a phosphate-buffered saline (pH 7.2) solution containing 0.3% Triton X-100, 3% normal horse serum, and 2% bovine serum albumin. Primary antibody (human p75 neurotrophin receptor, 1:60 000; Neomarkers, Fremont, California) was applied for 4 hours at approximately 25°C in phosphate-buffered saline containing 0.4% Triton X-100, 1% normal horse serum, and 1% bovine serum albumin. Sections were processed (ABC kit; Vector Laboratories, Burlingame, California) and developed with 2.5% nickel II sulfate, 0.05% 3,3-diaminobenzidine (Sigma-Aldrich Inc, St Louis, Missouri), and 0.005% hydrogen peroxide.19 Immunostained tissue sections were slide mounted but not coverslipped and were stored in phosphate buffer at 4°C.

SINGLE-CELL MICROASPIRATION, RNA AMPLIFICATION, AND ARRAY HYBRIDIZATION

Immunopositive neurons from the anterior NB subfield were aspirated using a micromanipulator and microcontrolled vacuum source (Eppendorf, Westbury, New York) attached to an inverted microscope (Nikon TE2000; Fryer, Huntley, Illinois).13,20,21 RNA amplification from NB neurons was performed using terminal continuation (TC) RNA amplification methods17,18 (http://cdir.rfmh.org/pages/ginsberglabpage.html). The final amplification step used complementary DNA (cDNA) made from individual neuronal mRNA as a template for in vitro transcription in the presence of T7 RNA polymerase and phosphorus P

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33 uridine triphosphate. Radiolabeled TC RNA probes were then hybridized to custom-designed cDNA arrays. Arrays were hybridized overnight at 42°C in a rotisserie oven and were washed sequentially in 2× saline sodium citrate (SSC) buffer–0.1% sodium dodecyl sulfate (SDS), 1× SSC–0.1% SDS, and 0.5× SSC–0.1% SDS for 20 minutes each at 42°C.13,20,21 Arrays were placed in a phosphor screen for 24 hours and were developed on a phosphor imager (GE Healthcare, Piscataway, New Jersey).

CUSTOM-DESIGNED cDNA ARRAY PLATFORMS AND DATA COLLECTION

Array platforms consisted of 1 µg of linearized cDNA purified from plasmid preparations adhered to high-density nitrocellulose (Hybond XL, GE Healthcare). Approximately 576 cDNAs and/or expressed sequence tags (ESTs) were used on the array platform. The human ESTs encoding specific nAChRs and mAChRs were selected using the UniGene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene) to identify individual subunits with minimal cross-hybridization based on sequence homology to the original cDNAs from rodents22,23 as described previously.21,24 Each AChR EST was specific for the respective individual subunit, and each EST is sequence verified to be 100% identical to the wild-type EST with the UniGene database. Expression of TC-amplified RNA bound to each linearized cDNA was expressed as a ratio of the total hybridization signal intensity of the array (ie, global normalization).23 Hybridization signal intensity is quantified by subtracting background using empty vector (pBluescript; Stratagene, La Jolla, California). Expression of TC-amplified RNA bound to each linearized cDNA was expressed as a ratio of the total hybridization signal intensity of the array (ie, global normalization).23 Data analysis generated an expression profile of relative changes in mRNA levels among cholinergic NB neurons isolated from different clinical groups.

REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

Real-time quantitative polymerase chain reaction (qPCR) was performed on unfixed microdissected frozen anterior NB and caudate tissue from 11 NCI cases, 9 MCI cases, and 10 mild to moderate AD cases using qPCR primers specific for human α4 and α7 nAChRs and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (SuperArray, Frederick, Maryland). Samples were loaded in triplicate on 96-well plates and were analyzed using SYBR green reporter dye on a DNA engine (Opticon 2; Bio-Rad, Hercules, California). Standard curves and cycle threshold were measured using standards obtained from human postmortem brain RNA. For graphical representation, cycle threshold values were converted to signal intensity values using commercially available software (Easy Engine, Bio-Rad).

STATISTICAL ANALYSIS

Demographic variables (Table) were compared among clinical diagnostic groups using a Kruskal-Wallis test or Fisher exact test, with Bonferroni-type correction for pairwise comparisons. Expression levels of AChR mRNAs were clustered and displayed using a bioinformatics and graphics software package (GeneLinker Gold; Predictive Patterns, Kingston, Ontario, Canada).13,20,23 A false discovery rate-controlling procedure23 was used to reduce type I errors due to the large number of genes analyzed simultaneously. Relative changes in hybridization signal intensity of individual mRNAs and qPCR were analyzed using 1-way analysis of variance with post hoc Newman-Keuls analysis. The association between gene expression levels and clinical or neuropathological variables was evaluated via mixed-models repeated-measures analyses.24 The level of significance was set at P < .01.

A total of 174 single cholinergic NB neurons were analyzed by subtracting background using empty vector (pBluescript; Stratagene, La Jolla, California). Expression of TC-amplified RNA bound to each linearized cDNA was expressed as a ratio of the total hybridization signal intensity of the array (ie, global normalization).23 Data analysis generated an expression profile of relative changes in mRNA levels among cholinergic NB neurons isolated from different clinical groups.
GAPDH

CHRNA7 expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) expression levels for quantitative analysis. CHRNA4 and CHRNA7 expression levels were normalized to GAPDH expression levels for quantitative analysis.

Figure 2. Quantitative polymerase chain reaction validation of CHRNA7 and CHRNA4 expression profiling. A, CHRNA7 messenger RNA in anterior nucleus basalis (NB). *P < .01, mild to moderate Alzheimer disease (AD) compared with no cognitive impairment (NCI) and mild cognitive impairment (MCI). B, CHRNA4 in anterior NB. C, CHRNA7 in striatum. CHRNA4 and CHRNA7 expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) expression levels for quantitative analysis. CHRNA4 and CHRNA7 expression levels were normalized to GAPDH expression levels for quantitative analysis.

al Cognitive Score (P < .001 for both). A statistically significant association was also found between increasing α7 nAChR mRNA levels and a higher likelihood of AD by the National Institute on Aging and the Reagan Institute Working Group criteria (P = .01). No association was found between α7 nAChR levels and Braak stage (P = .11).

Array data were validated by qPCR analysis of α4 and α7 nAChR mRNA levels in frozen anterior NB and caudate nucleus tissue from the same cases. Similar to the single NB neuron expression profiling studies, α7 nAChR mRNA levels were statistically significantly increased in NB samples from mild to moderate AD cases relative to NCI and MCI cases (F2,20 = 5.396, P < .01) (Figure 2A), whereas α4 nAChR mRNA levels were similar among the diagnostic groups (Figure 2B). In the caudate nucleus, an area enriched in cholinergic neurons but unaffected in AD,4 α7 nAChR mRNA levels were unchanged in mild to moderate AD cases compared with NCI and MCI cases (Figure 2C); α4 nAChR mRNA was not detected. The α7 nAChR primer set revealed no cross-reactivity with α4 nAChRs (cycle threshold < 10 using 10 ng of α7 nAChR plasmid as input cDNA; cycle threshold > 40 using 10 ng of α4 nAChR plasmid as input cDNA).

We report the novel finding that individual cholinergic NB neurons up-regulate the expression of α7 nAChR subunit mRNA in mild to moderate AD but not in MCI. These subunits are likely expressed as homomeric α7 nAChR assemblies, which combined with heteromeric α4β2 assemblies constitute the 2 major nAChR subtypes expressed in brain.3 Subunit expression for the α4β2 subtype was unchanged across clinical diagnoses. A principal strength of the present single-cell profiling approach is that the target AChR cDNA sequences on the custom arrays are nonoverlapping. This provides optimal mAChR subtype and nAChR subunit specificity and sensitivity and avoids potential confounds inherent in studies in which, for instance, nAChRs are detected by radioligands that cannot discriminate between individual subunits.3

The probable increase in α7 nAChRs (as indexed by increased message) in AD may regulate basocortical cholinergic tone through presynaptic or postsynaptic mechanisms within NB neurons before their frank degeneration in the later stages of AD. In human brain, α7 nAChR–specific hybridization probes and iodine 1125-α-bungarotoxin label NB neurons,9 suggesting that α7 nAChRs within cholinergic cells are localized to postsynaptic somatodendritic compartments. Cholinergic inputs to cholinergic NB perikarya have been identified in primate brain,3 while nicotine and nicotinic agonists depolarize these cells in the presence of tetrodotoxin.26,27 Hence, increased postsynaptic α7 nAChR expression may facilitate feed-forward cholinergic activity within NB cortical projection neurons in AD. Increased nicotinic transmission may also result in altered gene expression through the transcription factor cyclic adenosine monophosphate–dependent binding protein (CREB),28 which promotes cholinergic neuron survival.29 Alternatively, whereas the presence of α7 nAChRs on cholinergic axons within the cortex remains controversial,30,31 presynaptic α7 nAChRs have been shown to stimulate ACh release.32 This suggests that increased NB neuron α7 nAChR expression could stimulate autoreceptor-mediated ACh release within cortical projection sites during AD progression in an effort to increase a failing cholinergic signal. Since the anterior NB cholinergic neurons project to the frontal cortex,33 an increase in presynaptic α7 nAChRs in these
neurons may account for the stability of α7 nAChR levels in the frontal cortex compared with its decrease in other cortical areas in AD. This putative plasticity response in NB neurons is reminiscent of the increases in α7 nAChRs observed in other limbic structures, including the entorhinal cortex and hippocampus as well as in peripheral leukocytes in AD. This latter observation, combined with our present data showing that NB α7 nAChR mRNA expression is inversely associated with Global Cognitive Score and Mini-Mental State Examination score, suggests that increases in this receptor may serve as a marker for AD progression.

Despite potential beneficial roles for increased NB neuronal α7 nAChR expression in AD, evidence suggests that increased α7 nAChR expression contributes to cellular degeneration. Notably, α7 nAChRs bind or interact with β-amyloid (Αβ) precursor protein and Aβ peptides, which form the nidus of senile plaques in AD. Although α7 nAChR activation can be protective against Aβ neurotoxicity, competitive Aβ binding to α7 nAChRs may inhibit the action of this receptor in promoting long-term potentiation or activation of CREB. Furthermore, cell surface Aβ-α7 nAChR interactions may promote Aβ endocytosis, producing intraneuronal Aβ accumulation, with possible subsequent plaque formation. Therefore, increased NB neuronal α7 nAChR expression may serve as an explanatory neuroprotective response that is offset by aberrant Aβ-α7 nAChR interactions, leading to cholinergic dysfunction.

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Author Contributions: Study concept and design: Counts, Che, DeKosky, Ginsberg, and Mufson. Acquisition of data: Counts, He, Che, and Ginsberg. Analysis and interpretation of data: Counts, Ikonomovic, DeKosky, Ginsberg, and Mufson. Drafting of the manuscript: Counts, Ginsberg, and Mufson. Critical revision of the manuscript for important intellectual content: He, Che, Ikonomovic, DeKosky, and Ginsberg. Statistical analysis: Counts. Obtained funding: Counts, Ikonomovic, DeKosky, Ginsberg, and Mufson. Administrative, technical, and material support: Counts, He, Che, DeKosky, Ginsberg, and Mufson. Study supervision: Counts, Ginsberg, and Mufson.

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