Superior Frontal Cortex Cholinergic Axon Density in Mild Cognitive Impairment and Early Alzheimer Disease

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Background: Loss of cortical choline acetyltransferase (ChAT) activity contributes to end-stage Alzheimer disease (AD) dementia. In general, ChAT activity levels are stable in the neocortex in mild to moderate AD (mAD) and there is a selective up-regulation in the superior frontal cortex (SFC) in mild cognitive impairment (MCI), indicating a transient, region-specific cholinergic neuroplastic response.

Objective: To assess whether a proliferation of cholinergic axons underlies increased ChAT activity levels in the SFC in subjects with MCI.

Design: Stereologic principles were applied to assess the density of ChAT-immunoreactive fibers and axon varicosities in SFC tissue obtained postmortem from subjects with no cognitive impairment, MCI, and mAD.

Subjects: Thirty-six subjects enrolled in the Religious Orders Study, with records of annual clinical evaluation for frontal lobe specific and global cognitive functions.

Results: Compared with the group with no cognitive impairment, SFC ChAT-immunoreactive fiber and axon varicosity densities were not altered in the MCI group but were significantly reduced in the group with mAD and correlated with impaired frontal lobe and global cognitive function.

Conclusions: The lack of an increase in cholinergic axonal innervation of the SFC in MCI suggests that structural reorganization of cholinergic profiles is not the mechanism underlying the transient cholinergic plasticity reported in this region. Furthermore, the stability of cholinergic enzyme activity in mAD is likely the result of a biochemical up-regulation of ChAT protein or enzyme activity levels in the SFC, compensating for decreased regional cholinergic fibers and axon varicosities.

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METHODS

SUBJECTS

Tissue was obtained postmortem from 36 participants in the Religious Orders Study, a longitudinal clinicopathologic study of aging and AD in Catholic nuns, priests, and brothers. Clinical evaluation was based on tests obtained within 12 months before death and consisted of a global cognitive score and assessment of frontal lobe function using a composite z score from the following 5 cognitive tests: Consortium to Establish a Registry for Alzheimer's Disease (CERAD) 3-9.
mer’s Disease (CERAD) Word List, Immediate Recall Trials 1 through 3; Digits Backward; Digits Ordering; Symbol Digit Modalities Test, Oral Version; and Standard Progressive Matrices. Subjects were categorized as having NCI (n=11), MCI (n=11), or mAD (n=14) (Table). Subjects with MCI had cognitive impairment insufficient to meet criteria for dementia. The AD group represented subjects with mild to moderate dementia based on their Mini-Mental State Examination (MMSE) scores (Table). The diagnosis of AD dementia was based on standard criteria. The study was approved by the human investigations committees of Rush University Medical Center, Chicago, Illinois, and the University of Pittsburgh, Pittsburgh, Pennsylvania.

ChAT IMMUNOHISTOCHEMISTRY

Tissue blocks containing the middle third of the SFC (Brodmann area 9) were dissected as previously described. Each case was coded and assigned neuropathologic diagnoses that included Braak staging of neurofibrillary tangle pathologic findings, CERAD, and NIA-Reagan (National Institute on Aging–Reagan Institute Working Group) 1997 diagnoses (Table). The distribution of AD dementia was based on standard criteria. The study was approved by the human investigations committees of Rush University Medical Center, Chicago, Illinois, and the University of Pittsburgh, Pittsburgh, Pennsylvania.

Table. Demographic, Cognitive, and Pathologic Characteristics by Clinical Diagnostic Category

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NCI (n=11)</th>
<th>MCI (n=11)</th>
<th>AD (n=14)</th>
<th>Total (N=36)</th>
<th>P Value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at death, y</td>
<td>84.0±4.9 (74 to 92)</td>
<td>84.1±6.7 (75 to 97)</td>
<td>87.3±6.0 (82 to 100)</td>
<td>85.3±5.7 (82 to 100)</td>
<td>.30</td>
</tr>
<tr>
<td>Male sex, No. (%)</td>
<td>6 (55)</td>
<td>4 (36)</td>
<td>3 (21)</td>
<td>13 (36)</td>
<td>.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Educational achievement, y</td>
<td>18.8±2.5 (16 to 22)</td>
<td>17.1±3.6 (8 to 21)</td>
<td>17.6±2.5 (6 to 24)</td>
<td>17.6±3.4 (6 to 24)</td>
<td>.40</td>
</tr>
<tr>
<td>Apolipoprotein e&lt;sub&gt;4&lt;/sub&gt; allele, No. (%)</td>
<td>3 (27)</td>
<td>4 (36)</td>
<td>7 (50)</td>
<td>14 (39)</td>
<td>.50&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Postmortem interval, h</td>
<td>7.4±4.3 (2.2 to 16)</td>
<td>7.0±4.8 (3 to 16)</td>
<td>6.6±2.9 (3 to 11.5)</td>
<td>6.8±3.8 (2.2 to 16)</td>
<td>.80</td>
</tr>
<tr>
<td>MMSE score</td>
<td>27.1±1.4 (25 to 30)</td>
<td>25.8±1.8 (22 to 28)</td>
<td>21.1±3.4 (14 to 25)</td>
<td>24.8±3.3 (14 to 30)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Global Cognitive Scale score</td>
<td>0.4±0.3 (−0.1 to 1.1)</td>
<td>0.1±0.3 (−0.3 to 0.7)</td>
<td>−0.5±0.3 (−1.5 to −0.1)</td>
<td>−0.05±0.5 (−1.5 to 1.1)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Frontal lobe function composite z score</td>
<td>0.5±0.2 (−0.1 to 1.4)</td>
<td>0.2±0.1 (−0.3 to 1.0)</td>
<td>−0.4±0.1 (0.1 to −1.8)</td>
<td>0.04±0.01 (−1.8 to 1.4)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Abbreviations: AD, Alzheimer disease; CERAD, Consortium to Establish a Registry for Alzheimer’s Disease; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; NCI, no cognitive impairment; NIA-Reagan, National Institute on Aging–Reagan Institute Working Group.

<sup>a</sup>Values are given as mean±SD (range) unless otherwise indicated.

<sup>b</sup>Comparison by diagnosis group. Values obtained by 1-way analysis of variance unless otherwise indicated.

<sup>c</sup>Fisher exact test.

<sup>d</sup>Kruskal-Wallis test.

Densities of ChAT-ir fibers and associated varicosities were determined on 2 tissue sections chosen randomly from the processed series using a stereologic sampling-based method. Immunohistochemistry was performed using a polyclonal goat anti-ChAT antibody (1:100, antihuman placental enzyme, code AB144P, lot No. 23031288; Chemicon International, Temecula, California). The specificity of this antibody for human ChAT has been reported previously.

QUANTIFICATION OF ChAT-ir FIBER AND AXON VARICOSITIES

Chromogen-based immunohistochemistry was performed using a polyclonal goat anti-ChAT antibody (1:100, antihuman placental enzyme, code AB144P, lot No. 23031288; Chemicon International, Temecula, California). The specificity of this antibody for human ChAT has been reported previously.
12 deep ROIs per case. Under the immunolabeling conditions used in this study, the ChAT antibody penetrates throughout the full depth of the section. In all images were captured at a 0-axis level that resulted in the greatest concentration of fibers in clear focus. Each imaged ROI was superimposed with a cytochrome oxidase or point-counting grid for analyses of fibers or axon varicosities, respectively. In each ROI, we counted intersections of cycloids with all ChAT-ir fibers, regardless of their size. For the axon varicosity analysis, ChAT-ir swellings associated with ChAT-ir fibers and that were in clear focus were counted in 5 randomly chosen grid boxes within the original ROI.

STATISTICAL ANALYSIS

Results of ChAT-ir fiber and axon varicosity density analyses were compared among diagnostic groups using 1-way analysis of variance, with Tukey’s method for post hoc comparisons. One-way analysis of variance and the Fisher exact and Kruskal-Wallis tests were applied to the comparison of clinical, demographic, and pathologic variables across diagnostic groups. Associations of ChAT-ir fiber and axon varicosity data with clinical, demographic, and neuropathologic variables were examined using the Spearman rank correlation or the Wilcoxon rank sum test. The level of statistical significance for all tests was set at .01 (2-sided).

RESULTS

There were no differences in postmortem interval, age, years of educational achievement, sex, or presence of the apolipoprotein ε4 allele among the 3 diagnostic groups (NCI, MCI, and mAD; Table). The groups differed in Braak scores (Table), and there was a trend toward a difference in NIA-Reagan and CERAD diagnoses (Table). For tests of general cognition (MMSE and Global Cognitive Score; Table) and for frontal lobe function, the NCI and MCI groups scored significantly better than the mAD group (Table).

In all subjects examined, ChAT-ir fibers and axon varicosities in the SFC were observed as a network of long processes with multiple swellings throughout the gray matter, with greater densities in more superficial (laminae II and III) compared with deep gray matter (laminae V and VI) (Figure 1). ChAT-ir fiber and axon varicosity densities in the SFC were comparable between the NCI and MCI groups but were significantly reduced in the mAD group ($P < .01$), with the greatest differences in the SFC superficial laminae (Figure 1 and Figure 2). The results remained unchanged after correcting for cortical laminar thickness (data not shown) or when the AD group included only those subjects with an MMSE score of 20 or higher (n=11) (data not shown). ChAT-ir fibers often contained abnormally enlarged varicosities in subjects with mAD (Figure 3).

Overall, ChAT-ir fiber and axon varicosity densities were significantly correlated (superficial gray matter, $r=0.83$; deep gray matter, $r=0.78$; both $P < .001$); similarly, ChAT-ir profile densities in superficial vs deep laminae correlated strongly (fibers, $r=0.95$; varicosities, $r=0.82$; both $P < .001$). The following correlative analyses of ChAT-ir profiles with demographic data, pathologic findings, and cognitive function are based on total (combined superficial and deep lamina) values. No association was found between ChAT-ir fiber and axon varicosity densities with any demographic variable. Both ChAT-ir profiles correlated directly with scores on the MMSE (fibers, $r=0.57$, $P < .001$; varicosities, $r=0.53$, $P < .001$) and Global Cognitive Score (fibers, $r=0.52$, $P < .001$; varicosities, $r=0.50$, $P < .001$) and with the composite z score for frontal lobe function (fibers, $r=0.54$, $P < .001$; varicosities, $r=0.54$, $P < .001$). There was a trend toward an inverse correlation of ChAT-ir fiber and varicosity densities with frequencies of neuritic amyloid plaques (fibers, $r=-0.26$, $P = .06$; varicosities, $r=-0.23$, $P = .03$) and diffuse amyloid plaques (fibers, $r=-0.13$, $P = .22$; varicosities, $r=-0.35$, $P = .02$) in the SFC.

COMMENT

The present study demonstrates that cholinergic projections to the SFC are not altered in MCI but are markedly reduced in early stages of AD. The distribution of cholinergic axonal profiles in the SFC, with greater fiber density in superficial laminae across all clinical diagnostic groups, supports previous morphologic and laminar microchemical analyses of ChAT activity in the SFC. Ultrastructural electron microscopic studies revealed that ChAT-ir fibers and varicosities are thin unmyelinated axons and boutons containing synaptic vesicles that make presynaptic perikaryal contacts. Because as many as 67% of cortical cholinergic axon varicosities make synaptic contacts, the reduction of ChAT-ir...
fibers and varicosities observed in the SFC in subjects with mAD in the present study likely reflects loss of cholinergic synaptic specializations.

Stable densities of SFC ChAT-ir fibers and axon varicosities in MCI and their loss in mAD was not expected in light of our previous biochemical findings of increased ChAT activity in this region in subjects with MCI compared with subjects with NCI or mAD from the same cohort. Together, these observations suggest that in MCI, plasticity of the cholinergic system occurs through biochemical up-regulation of ChAT enzyme activity rather than an increase (sprouting) in fiber projections to the SFC. In mAD, SFC ChAT-ir axonal projections are reduced (present study), whereas biochemically measured ChAT enzyme activity is comparable to levels observed in NCI, indicating that a biochemical up-regulation also occurs in mAD. This agrees with the suggestion that during AD progression, synapse loss precedes loss of ChAT activity in the SFC. Increased ChAT activity and drebrin protein levels in MCI and up-regulation of the high-affinity choline transporter in AD indicate that multiple compensatory mechanisms are recruited to maintain cholinergic neural transmission in the frontal cortex in MCI and mAD. Similar compensatory mechanisms may occur in the hippocampus, which

Figure 2. Box plots of choline acetyltransferase immunoreactive (ChAT-ir) axonal fibers and varicosity densities (arbitrary units) in the superior frontal cortex in subjects in the groups with no cognitive impairment (NCI), mild cognitive impairment (MCI), and mild to moderate Alzheimer disease (mAD). Black line indicates the median.

Figure 3. High-power photomicrographs show choline acetyltransferase axonal varicosities in the superior frontal cortex in individuals with no cognitive impairment (A), mild cognitive impairment (B), and mild to moderate Alzheimer disease (C). In mild to moderate Alzheimer disease, axonal varicosities in the superior frontal cortex occur less frequently and often are abnormally enlarged. Images were obtained under brightfield illumination and grayscale inverted for purposes of illustration. Scale bar indicates 15 µm.
is also characterized by increased ChAT activity levels in MCI.\(^7\)

The preservation of ChAT immunoreactivity,\(^17\) despite changes in high-affinity trkA\(^11\) and low-affinity p75\(^{NTR}\) neurotrophin receptors\(^12\) in cholinergic basal forebrain neurons suggests that the increase in ChAT enzyme activity levels in MCI and its stability despite loss of cholinergic axons and varicosities in mAD results from altered metabolic status of cholinergic basal forebrain neurons\(^26\) that project to the SFC. Alternatively, increased ChAT activity levels could result from impaired axonal transport reflected by enlarged ChAT-ir axon varicosities in the SFC in subjects with mAD. Similar abnormal ChAT-ir axon varicosities were described in previous studies of AD,\(^27,28\) and their functionality remains to be determined.

There were several methodologic limitations in this study. Although our sampling approach was based on unbiased stereologic principles, the entire SFC was unavailable for evaluation. Therefore, a systematic uniformly random sampling of the entire SFC was impossible. This also prevented estimates of SFC volume and precluded conversion of density estimates into estimates of total numbers of fibers and axon varicosities. There is no stereologic method for uniform sampling of cortical structures for laminar analyses. Any biases associated with our sampling approach are limited, based on the assumption that fiber innervation and the cytoarchitecture of the SFC are homogeneous if consistently sampled perpendicular to the pial surface, as in the present study. Potential biases were further minimized by maintaining strict sampling and analytical procedures, enabling fair comparisons among the 3 clinical diagnostic groups.

**CONCLUSIONS**

Stability of the SFC cholinergic infrastructure and increased ChAT activity levels in MCI could explain why cholinesterase inhibitors are not as effective in these patients as one would expect.\(^29\) Reduced densities of SFC ChAT-ir fibers and varicosities in mAD may hinder the ability of the cholinergic system to up-regulate ChAT activity sufficiently in more advanced disease stages. Failure of this compensatory mechanism may contribute to clinical deterioration during the transition from MCI to mAD. The goal of future therapies should be to sustain neocortical cholinergic afferents by supporting the viability and functionality of cholinergic basal forebrain neurons\(^30\) in an attempt to prevent or delay the onset of clinical dementia or to impede further decline in individuals with the earliest signs of cognitive dysfunction.

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Announcement

Calendar of Events: A New Web Feature

On the new Calendar of Events site, available at http://pubs.ama-assn.org/cgi/calendarcontent and linked off the home page of the Archives of Neurology, individuals can now submit meetings to be listed. Just go to http://pubs.ama-assn.org/cgi/cal-submit/ (also linked off the Calendar of Events home page). The meetings are reviewed internally for suitability prior to posting. This feature also includes a search function that allows searching by journal as well as by date and/or location. Meetings that have already taken place are removed automatically.