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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Delicits in cortical cholinergic enzyme activity correlate with cognitive impairment in Alzheimer disease (AD).1-4 In early AD (mild to moderate AD [mAD]), cholinergic enzyme activity levels are preserved in all cortical regions.5-7 Subjects with mild cognitive impairment (MCI) exhibit increased choline acetyltransferase (ChAT) activity levels in the superior frontal cortex (SFC),7 indicating a region-specific pre-synaptic cholinergic plasticity response before the onset of AD dementia. In addition, subjects with MCI have elevated levels of drebrin, a postsynaptic dendritic spine marker, in the SFC,8 indicating that changes occur both pre-synaptically and post-synaptically in this region during preclinical AD. We hypothesized that cholinergic plasticity in the SFC in subjects with MCI reflects increases in ChAT-containing fiber and axon varicosities densities.

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.7,9 Clinical evaluation was based on tests obtained within 12 months before death and consisted of a global cognitive score9 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) cognitive battery, the Trail Making Test, the Digit Symbol Substitution Test, the California Verbal Learning Test, and the Mattis Dementia Rating Scale. Mixed effects models were used to control for the confounding effects of sex, education, and cognitive decline.
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, \(^{13}\) CERAD, \(^{14}\) and NIA-Reagan (National Institute on Aging–Reagan Institute Working Group) 1997 diagnoses (Table) by a board-certified neuropathologist. Cases were excluded if tumors, large strokes, multiple lacunar infarctions, signs of encephalitis, or pathologic features of Parkinson disease were detected. Chromogen-based immunohistochemistry was performed \(^{15,17}\) 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.\(^{16}\)

### QUANTIFICATION OF ChAT-ir FIBER AND AXON VARICOSITIES

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. Inasmuch as it was impossible to determine the total volume of Brodmann area 9 because of the limited number of sections available, density measurements were expressed in arbitrary units. Previous studies of the neocortex in subjects with AD and neurologically healthy subjects demonstrated lamina-specific variations in ChAT-ir fiber densities\(^{18}\) and ChAT enzyme activity.\(^{19}\) Accordingly, ChAT-ir fibers and axon varicosities were quantified separately in the superficial and deep laminae of the SFC, determined on adjacent matching tissue sections counterstained with cresyl violet to aid in cytoarchitecture analysis.\(^{20}\) For each section, 6 evenly spaced regions of interest (ROIs) (×40 magnification optical fields positioned parallel to the cortical surface) were captured in the superficial gray matter (laminae II and III) and 6 corresponding ROIs in the deep gray matter (laminae V and VI). This resulted in up to 12 superficial and...
cosities, respectively. In each ROI, we counted intersections
cloid or point-counting grid for analyses of fibers or axon vari-
in clear focus. Each imaged ROI was superimposed with a cy-
z-axis level that resulted in the greatest concentration of fibers
4 randomly chosen grid boxes within the original ROI.
ChAT-ir fibers and that were in clear focus were counted in
the axon varicosity analysis, ChAT-ir swellings associated with
of cycloids with all ChAT-ir fibers, regardless of their size. For
50 µm.
illumination and inverted for purposes of illustration. Scale bar indicates
exhibit a marked decline in mAD. Images were obtained under brightfield
impairment (MCI) (B and E), or mild to moderate Alzheimer disease (mAD)
individuals with no cognitive impairment (NCI) (A and D), mild cognitive
fibers and varicosities in superficial (A-C) and deep (D-F) laminae in
processes with multiple swellings throughout the gray
matter, with greater densities in more superficial (lami-
nae II and III) compared with deep gray matter (lami-
nae V and VI) (Figure 1). ChAT-ir fiber and axon vari-
cosity 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 cor-
tical 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 fi-
ers 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 $P < .001$); simi-
larly, ChAT-ir profile densities in superficial vs deep lam-
nae correlated strongly (fibers, $r = 0.95$; varicosities,
$r = 0.82$; both $P < .001$). The following correlative analy-
yses of ChAT-ir profiles with demographic data, patho-
logic findings, and cognitive function are based on total
(combined superficial and deep lamina) values. No as-
soocation was found between ChAT-ir fiber and axon var-
cosity 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 com-
posite $z$ score for frontal lobe function (fibers, $r = 0.54$,
$P < .001$; varicosities, $r = 0.54$, $P < .001$). There was a trend
ward toward an inverse correlation of ChAT-ir fiber and vari-
cosity densities with frequencies of neuritic amyloid
plaques (fibers, $r = -0.26$, $P = .06$; varicosities, $r = -0.33$,
$P = .03$) and diffuse amyloid plaques (fibers, $r = -0.13$,
$P = .22$; varicosities, $r = -0.35$, $P = .02$) in the SFC.

STATISTICAL ANALYSIS
Results of ChAT-ir fiber and axon varicosity density analyses
were compared among diagnostic groups using 1-way analy-
ysis of variance, with Tukey’s method for post hoc compar-
isons. One-way analysis of variance and the Fisher exact
Kruskal-Wallis tests were applied to the comparison of clini-
cal, 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 Wil-
coxon 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 dif-
fERENCE in NIA-Reagan and CERAD diagnoses (Table).
For tests of general cognition (MMSE and Global Cogni-
tive 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 vari-
cosities in the SFC were observed as a network of long
processes with multiple swellings throughout the gray
matter, with greater densities in more superficial (lami-
nae II and III) compared with deep gray matter (lami-
nae V and VI) (Figure 1). ChAT-ir fiber and axon vari-
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$P = .22$; varicosities, $r = -0.35$, $P = .02$) in the SFC.

COMMENT
The present study demonstrates that cholinergic projec-
tions to the SFC are not altered in MCI but are mark-
edly reduced in early stages of AD. The distribution of
cholinergic axonal profiles in the SFC, with greater fi-
bber density in superficial laminae across all clinical di-
agnostic groups, supports previous morphologic and
laminar microchemical analyses of ChAT activity in the
SFC. Ultrastructural electron microscopic studies re-
vealed that ChAT-ir fibers and varicosities are thin or
myelinated axons and boutons containing synaptic
vesicles that make presynaptic perikaryal contacts. Be-
cause as many as 67% of cortical cholinergic axon var-
cosities make synaptic contacts, the reduction of ChAT-ir
fibers in MCI and AD suggests that the ChAT-ir
cholinergic projection system is disproportionately dam-
aged in these disorders. The present findings could be
useful in the identification of potential therapeutic targets
in MCI and AD.
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
is also characterized by increased ChAT activity levels in MCI.7
The preservation of ChAT immunoreactivity,17 despite changes in high-affinity trkA11 and low-affinity P75NTR neurotrophin receptors12 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 neurons26 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.

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 neurons29 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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REFERENCES

17. Gilmore ML, Erickson JD, Varoqui H, et al. Preservation of nucleus basalis neurons containing choline acetyltransferase and the vesicular acetylcholine trans-