Regulatory changes in presynaptic cholinergic function assessed in rapid autopsy material from patients with Alzheimer disease: Implications for etiology and therapy

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ABSTRACT Brain regions from patients with or without Alzheimer disease (AD) were obtained within 2 hr of death and examined for indices of presynaptic cholinergic function. Consistent with loss of cholinergic projections, cerebral cortical areas involved in AD exhibited decreased choline acetyltransferase (acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) activity. However, remaining nerve terminals in these regions displayed marked up-regulation of synaptosomal high affinity [³H]choline uptake, a result indicative of relative cholinergic hyperactivity. As choline uptake is also rate-limiting in acetylcholine biosynthesis, these findings have implications for both therapy and identification of causes contributing to neuronal death in AD.

Alzheimer disease (AD), the leading cause of dementia and currently the fourth leading cause of death in the United States (1), is characterized by the abnormal presence of senile plaques and neurofibrillary tangles in the central nervous system. Neurochemically, the loss of cholinergic projections to the cerebral cortex is the hallmark characteristic of AD and has served as a focus for studies of its etiology and therapy (2–9). Dietary supplementation with neurotransmitter precursors represents a useful approach to the management of neurodegenerative diseases. The most prominent example is Parkinson disease, in which many of the symptoms of dopaminergic deficiency can be offset with L-dopa (10). In contrast, choline supplementation has proven to be only marginally successful in treating AD (2–9), suggesting either that cholinergic presynaptic function may be affected over and above the loss of terminals or, alternatively, that the clinical dementia is not due solely to cholinergic dysfunction (11–13).

Acetylcholine synthesis proceeds via the active, high-affinity uptake of choline by the nerve terminal and subsequent conversion to acetylcholine by the enzyme choline acetyltransferase (ChAT; acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6). The uptake step is particularly important in that it is both rate-limiting and subject to regulation by neuronal impulse activity (14–16). However, this step has not generally been evaluated in studies with human tissues because fresh material is required to isolate viable nerve terminal particles (synaptosomes) that will maintain their uptake characteristics; the up-regulation of choline uptake associated with neural stimulation decays particularly rapidly postmortem (17, 18). The current study measures ChAT activity and synaptosomal [³H]choline uptake obtained from brain regions of AD and non-AD patients less than 2 hr after death. Because choline uptake, but not ChAT, is responsive to nerve stimulation, we have also assessed the uptake/ChAT ratio as an index of activity corrected for the relative loss of nerve terminals caused by AD.

EXPERIMENTAL PROCEDURES

Patient Selection and Diagnostic Criteria. Thirty-one patients from the Rapid Autopsy Program were included in this study: 22 with AD (one with coexisting Parkinson disease and one with coexisting multi-infarct dementia), 3 with other dementias (chronic meningoencephalitis, Pick disease, severe nonspecific neuronal loss, and gliosis), 2 with motor neuron disease (amyotrophic lateral sclerosis), 1 with cerebral infarcts (not demented), and 3 who were neurologically normal (metastatic melanoma, lymphoma, metastatic colon carcinoma). None of the patients received anticholinergic or antihistaminic medications during the week prior to death. The AD group consisted of 15 females and 7 males 57–93 years old (80 ± 2 years; mean $\pm$ SE) and the non-AD group contained 3 females and 6 males 48–79 years old (69 ± 3 years); none of the AD vs. non-AD differences found in this study interacted significantly with age or sex (data not shown). The methods for patient recruitment into the program and the standard protocols for terminal care (including 100% oxygen by nasal prongs and oral or intravenous fluids) were approved by the Duke Institutional Review Board and have been described elsewhere (19, 20).

To meet the criteria for rapid autopsy, the postmortem delay from the time of death to chilling of tissues in ice-cold sucrose was under 2 hr (range, 30–112 min) and did not differ in the AD group (57 ± 2 min) compared to the non-AD group (63 ± 7 min). Approximately 1 ml of gray matter was removed from the superior frontal gyrus, the superior parietal lobule, the superior temporal gyrus, the occipital cortex (Brodmann's area 17), caudate, putamen, and rostral hippocampal formation. Adjacent sections from each neocortical area were submitted for histologic analysis. Paraffin sections were stained with hematoxylin and eosin with a Luxol fast blue counterstain for myelin, a silver stain for neuritic plaques and neurofibrillary tangles (21), and a Congo red stain for amyloid. Plaques and tangles were counted in three microscopic fields in each cortical and hippocampal section and are expressed as plaques or tangles per mm². The histologic diagnosis of AD met the criteria proposed by Khachaturian (22) and other diagnoses were made according to customary neuropathologic criteria (23). As examples of the clear delineation of the AD vs. non-AD populations, the AD group exhibited 50 ± 4 (mean $\pm$ SE) and 38 ± 4 plaques per mm² in frontal and temporal cortex, respectively, whereas the non-AD group had only 2 ± 2 and 1 ± 1 ($P < 0.0001$); tangles per mm² in the AD group were 4.5 ± 1.3 and 14 ± 4 in the same two cortical regions but were not detected (0 ± 0) in the non-AD group ($P < 0.002$).

Tissue Preparation and Biochemical Determinations. Tissues were homogenized (four up–down strokes in a smooth

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Abbreviations: AD, Alzheimer disease; ANOVA, analysis of variance; ChAT, choline acetyltransferase.

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glass homogenizer fitted with a Teflon pestle) in 9 vol of 0.32 M sucrose buffered at pH 7.2 with 50 μ M sodium phosphate. An aliquot was removed and diluted 1:3 for assessment of ChAT activity (24) and the P₂ synaptosome fraction (25) was used for studies of high affinity [³H]choline uptake (26).

For ChAT assays, tubes were prepared containing 30 μ l of tissue preparation, 0.6 μ g of bovine serum albumin, and final concentrations of 0.2% Triton X-100, 20 mM choline chloride, 0.4 mM [¹⁴C]acetyl CoA, 60 mM sodium phosphate, 200 mM NaCl, 16.5 mM MgCl₂, 1 mM EDTA, and 0.12 mM physostigmine, buffered at pH 7.9 in a total vol of 60 μ l. Blanks contained water instead of tissue preparation. Samples were preincubated for 15 min on ice, transferred to a 37°C water bath for 30 min, and labeled acetylcholine was extracted by standard techniques (24).

Synaptosomes were prepared according to Kirksey *et al.* (25). In brief, the sucrose homogenates were sedimented at 1000 $\times$ g for 10 min to remove nuclei and cell debris and the supernatant solution was sedimented at 11,000 $\times$ g for 10

min. The resulting pellet was resuspended (Teflon to smooth glass) in the original volume of sucrose buffer and this P₂ preparation was used for measurement of high-affinity [³H]choline uptake as described by Shelton *et al.* (26). Duplicate tubes were prepared for each tissue sample, containing a final concentration of 0.1 μ M [³H]choline, 0.1 ml of tissue preparation, and 0.9 ml of Elliot's artificial cerebrospinal fluid; blanks were assessed by addition of a final concentration of 0.8 μ M hemicholinium-3, a specific inhibitor of high-affinity choline uptake. Samples were incubated at 37°C for 4 min, after which uptake was terminated by chilling on ice and immediate addition of 3 ml of ice-cold medium. Labeled synaptosomes were then harvested and washed by vacuum filtration using a cellulose acetate filter (pore size, 0.45 μ m) covered by a glass fiber filter (Whatman GF/C). On the average, high-affinity choline uptake in the human preparations ranged from 1.5–2 times blank values in cortical regions up to 4–8 times blank values in caudate and putamen.

Reproducibility of biochemical assays was verified with a

FIG. 1. ChAT activity and high-affinity [³H]choline uptake into synaptosomes in neocortical regions (FCX, frontal cortex; PCX, parietal cortex; TCX, temporal cortex; OCX, occipital cortex) and in hippocampus (HIP), caudate (CAUD), and putamen (PUT) of rapid autopsy material from patients with or without AD. Statistical differences are indicated and reflect main effects attributable to diagnosis (two-way ANOVA). Thus, for example, for ChAT in cortical regions, the non-AD group has overall higher values than the AD group (main treatment effect), but it is not possible to distinguish the effect in one cortical region vs. another (no interaction of treatment and cortical region), and therefore these are not tested separately.

standard preparation of adult rat cerebral cortex run concurrently with each set of patient samples; in addition, protein was measured in the P_2 fraction to confirm consistent recovery of synaptosomes.

Data Evaluation and Statistics. Data are reported as means and standard errors. All variables were first subjected to a two-way analysis of variance (ANOVA; factors of diagnostic category and region); however, the global test revealed that data could be separated into neocortical regions (frontal, parietal, temporal, and occipital cortex) vs. areas less (hippocampus, caudate) or least (putamen) affected by AD (27–29). Where differences related to diagnosis (main effect or interaction of diagnosis and region) were identified, the ANOVA was then repeated with separation into neocortical vs. other regions; if diagnosis–region interactions were still detected, individual regions were evaluated for differences in the AD vs. non-AD group by Duncan's multiple range test.

For all tests, significance was assumed at the level of $P < 0.05$.

RESULTS AND DISCUSSION

In keeping with biochemical and histological assessments of material obtained after more prolonged postmortem delays (2–9), examination of ChAT activity after rapid autopsy confirmed a significant deficit in the enzyme in AD patients, with the effect confined to neocortical regions (Fig. 1). Notably, [3 H]choline uptake into cortical synaptosomes was not decreased in AD. In fact, values tended to be elevated relative to the non-AD group ($0.05 < P < 0.10$), with the lack of statistical significance attributable to relatively large variability. The uptake/ChAT ratio was significantly elevated only in cortical regions; it was below that of non-AD patients elsewhere.

FIG. 2. ChAT activity and high-affinity [3 H]choline uptake into synaptosomes, assessed as a ratio to values in putamen. Statistical differences are indicated for ChAT and uptake/ChAT and reflect main effects attributable to diagnosis (two-way ANOVA). Asterisks indicate individual regions where the non-AD and AD groups differ (Duncan's multiple range test), assessed post hoc only where ANOVA indicated a significant interaction of region and diagnosis; significance was $P < 0.01$ for CAUD ChAT relative to putamen, $P < 0.01$ for FCX choline uptake relative to putamen, and $P < 0.02$ for PCX choline uptake relative to putamen. Abbreviations are the same as in Fig. 6.

Some of the variability in enzyme activity and uptake probably results from individual patient differences in the proximate cause of death, length of the agonal state, age, and premortem medication history. Accordingly, we corrected the values by using putamen as an internal standard, since this region contained the highest enzyme activity and uptake, does not display activity-related changes in uptake (14–16), and is thought to be relatively uninvolved in AD (27, 28); although the caudate may be also relatively spared for cholinergic deficits in AD, there is some evidence for neuronal damage, which precludes considering it a “control” region (29). As shown in Fig. 2, the corrected decrease in ChAT activity in the AD group was still significant and confined to cortical regions. With the use of the putamen as an internal standard, [³H]choline uptake was statistically significantly elevated in AD frontal cortex and parietal cortex, and the uptake/ChAT ratio was increased across all four cortical regions to a greater extent and more consistently than choline uptake alone. Also, a significant increase in ChAT in caudate was seen, probably due to preferential sparing of cholinergic interneurons in the face of loss of noncholinergic cells in this region (29).

The results obtained for [³H]choline uptake are consistent with the view that regions exhibiting cholinergic neuronal loss in AD experience a profound up-regulation of neuronal activity in remaining neurons. To some extent, this may be viewed as a compensatory reaction to offset impaired synaptic function. However, the degree of stimulation is extreme: animals exposed to convulsant drugs typically demonstrate increases in uptake of $\approx 30\%$ (17, 18), whereas 2- to 4-fold increases were seen in AD. Prolonged neural activation of this magnitude could, in fact, contribute to metabolic damage and neuronal death (30). A similar metabolic difference may operate in evaluating rapid autopsy material vs. that obtained after conventional postmortem delays. The magnitude of the ChAT deficit seen here was smaller than generally found with standard autopsy material (2–9), suggesting that postmortem deterioration of enzyme activity over the longer time periods imposed by standard autopsy procedures, may be more rapid when AD is present. It is important to note that the measurements of [³H]choline uptake, and hence the conclusion of increased neural activity, can only be obtained with rapid autopsy material; although postmortem chilling of the brain permits basal uptake to be measured for more prolonged periods, the neural activity related component decays rapidly after death (17, 18). Within the 2-hr limit imposed in our studies, we did not note any significant postmortem time-related differences in [³H]choline uptake or ChAT across the AD and non-AD populations (no significant interaction of postmortem time and diagnosis).

These results also have implications for prospective therapeutic regimens in AD directed toward the cholinergic system. In the face of severe up-regulation of choline uptake by surviving neurons, the relative lack of efficacy of dietary choline therapy (2–9) suggests either that inadequate amounts of choline actually reach or have been used by the cholinergic neurons or, alternatively, that signal transmission (acetylcholine storage or release, postsynaptic signal transduction) is a more likely therapeutic target than is acetylcholine synthesis.

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