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Preservation of Nucleus Basalis Neurons Containing Choline Acetyltransferase and the Vesicular Acetylcholine Transporter in the Elderly with Mild Cognitive Impairment and Early Alzheimer's Disease

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ABSTRACT

Immunocytochemistry for choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAChT) was used to examine the expression of these linked cholinergic markers in human basal forebrain, including cases with early stages of Alzheimer's disease (AD). Previous neurochemical studies have measured decreased ChAT activity in terminal fields, but little change or even increased levels of VAChT. To determine total cholinergic neuron numbers in the nucleus basalis of Meynert (nbM), stereologic methods were applied to tissue derived from three groups of individuals with varying levels of cognition: no cognitive impairment (NCI), mild cognitive impairment (MCI), and early-stage Alzheimer's disease (AD). Both markers were expressed robustly in nucleus basalis neurons and across all three groups. On average, there was no significant difference between the number of ChAT- (210,000) and VAChT- (174,000) immunopositive neurons in the nbM per hemisphere in NCI cases for which the biological variation was calculated to be 17%. There was approximately a 15% nonsignificant reduction in the number of cholinergic neurons in the nbM in the AD cases with no decline in MCI cases. The number of ChAT- and VAChT-immunopositive neurons was shown to correlate significantly with the severity of dementia determined by scores on the Mini-Mental State Examination, but showed no relationship to apolipoprotein E allele status, age, gender, education, or postmortem interval when all clinical groups were combined or evaluated separately. These data suggest that cholinergic neurons, and the coexpression of ChAT and VAChT, are relatively preserved in early stages of AD. J. Comp. Neurol. 411:693–704, 1999.

Indexing terms: nucleus basalis; choline acetyltransferase; stereology; cholinergic; basal forebrain; dementia

Progressive degeneration of cholinergic basal forebrain (CBF) neurons in Alzheimer's disease (AD) is supported by several lines of evidence. Choline acetyltransferase (ChAT) activity is decreased up to 80% in postmortem tissue samples from the cortex and hippocampus of AD patients (e.g., see Perry et al., 1978; Wilcock et al., 1982; McGeer et al., 1984; Etienne et al., 1986). Cortical ChAT activity is similarly decreased in biopsy samples, suggesting that

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cholinergic dysfunction may occur early in the disease process (Bowen et al., 1982; Sims et al., 1983; DeKosky and Scheff, 1990; DeKosky et al., 1992). Consistent with the decreases in cortical ChAT activity, histopathologic studies have reported degeneration of the large cholinergic basal forebrain neurons of the nucleus basalis (Whitehouse et al., 1981; Wilcock et al., 1982; Tagliavini and Pilleri, 1983; McGeer et al., 1984; Arendt et al., 1985) which provide the primary source of cholinergic innervation to the entire cortex (Mesulam et al., 1983; Mesulam and Geula, 1988). For example, quantitative studies that used Nissl-stained tissue to mark cholinergic neurons have revealed up to a 90% reduction in basocortical cholinergic neurons in AD (Whitehouse et al., 1981). Moreover, cholinomimetic therapies that use acetylcholinesterase inhibitors or selective muscarinic agonists have been shown to improve cognitive function in some patients with AD (Farlow et al., 1992; Bodick, 1997).

During the past several years, numerous studies that used markers other than ChAT, have shown that the alterations to the basocortical cholinergic system are more complex than originally proposed. For example, the vesicular acetylcholine transporter (VAChT) which is responsible for the accumulation of acetylcholine in synaptic vesicles in cholinergic axon terminals is not severely altered in AD. In this regard, pharmacologic studies of VAChT in postmortem AD tissue or in vivo imaging studies by using vesamicol and its analogs, suggest that VAChT levels remain steady or are minimally decreased coincident with a severe decline in ChAT activity in cortical areas (Kulmala, 1985; Kish et al., 1990; Ruberg et al., 1990; Kuhl et al., 1996). The discordance between ChAT and VAChT is particularly surprising in light of the recent discovery that they are part of a single cholinergic gene locus with shared regulatory elements (for review see Eiden, 1998). Similarly, binding or transport studies of the high affinity choline uptake transporter (HAChT), another putative specific marker of cholinergic terminals that is responsible for recycling of choline into the cholinergic axon terminal, also revealed stable or increased levels HAChT in AD cortex (Slotkin et al., 1990, 1994). Moreover, there is evidence from experimental lesions in animals (Sofroniew et al., 1983) and from postmortem human studies (Pearson et al., 1983; Rinne et al., 1987; Allen et al., 1988; Vogels et al., 1990) to suggest that many cholinergic neurons shrink after injury or during the pathologic process rather than degenerate. Although controversial, down-regulation of ChAT could also account for the apparent loss of neurons observed in experimental paradigms and in AD (Perry et al., 1982; Naumann et al., 1994). Taken together, these observations suggest that cholinergic basal forebrain (CBF) neurons may be viable, albeit, dysregulated in AD.

There is increasing interest in understanding the changes that occur in the brain in normal aging and in the earliest stages of AD. Although normal aging is not usually associated with substantial cognitive dysfunction or neuropathology, there are frequent senile plaques and neurofibrillary tangles present in cases with very mild AD, in cases with mild cognitive impairment at high risk for progressing to AD, and even in some cognitively intact elderly (West et al., 1994; Price and Morris, 1999; Mufson et al., in press). A major tenet of AD is that there is a loss of cholinergic neurons within the nucleus basalis in this disease (Whitehouse et al., 1981). Whether this profound change, which is seen in end-stage patients, extends to individuals with mild AD is unknown. To clarify the extent of CBF neuronal degeneration in the early stages of the disease process, we compared the total number of neurons containing ChAT and VAChT within the nucleus basalis of individuals clinically classified as displaying either no cognitive impairment (NCI), mild cognitive impairment (MCI), or early AD. In the past, such investigations in more advanced cases have relied on traditional nonstereologic morphometric counting techniques, which may be biased, and/or use nonspecific markers to identify cholinergic neurons. The only published report that used modern stereologic techniques counted Nissl-stained neurons within the basal forebrain and reported a 30% decrease in the total number of "cholinergic" neurons (Vogels et al., 1990). However, the use of Nissl histochemistry as opposed to a cholinergic neuron-specific marker, confounds these observations because noncholinergic as well as cholinergic neurons may have been included in the final analysis. Therefore, the present study was undertaken to carefully assess the number of cholinergic neurons within the human nucleus basal by using two cholinergic-specific markers, ChAT and VAChT.

MATERIALS AND METHODS

Clinical evaluation

Subjects were participants in the Religious Orders Study, a longitudinal clinical-pathologic study of aging and Alzheimer's disease. From January of 1994 through June of 1998, 664 older Catholic nuns, priests, and brothers from 25 groups located in nine states enrolled in the study. Groups are located in Chicago and its metropolitan area, California, Indiana, Iowa, Kentucky, Minnesota, New York, Tennessee, and Wisconsin. Each participant agreed to an annual detailed clinical evaluation and brain donation at the time of death. The study was approved by the Human Investigation Committee of Rush-Presbyterian-St. Luke's Medical Center. Follow-up participation for the annual evaluations is 99% of survivors. Of 78 participants who have died, 71 (91%) had undergone brain autopsy. Included in the investigation are nine subjects meeting clinical criteria for AD, seven subjects with MCI, and six subjects with no evidence of cognitive impairment (NCI), who did not have a coexisting condition judged to be contributing to cognitive impairment. All subjects were also analyzed for apolipoprotein E (ApoE) genotype by using methods adapted from those described previously (Hixson and Vernier, 1990).

The clinical evaluation included a medical history, neurologic examination, neuropsychologic performance testing, and review of brain scan when available. The medical history included uniform, structured questions about cognitive decline, stroke, Parkinson's disease, head injury, tumor, depression, and other medical problems. All medications used by the participant within the previous two weeks of the examination were reviewed and classified. A uniform, structured neurologic examination was performed by trained nurse clinicians; it included an assessment for stroke (Goldstein and Samsa, 1997) and parkinsonian signs (Bennett et al., 1997). A battery of 21 cognitive tests was administered by trained neuropsychology technicians. Cognitive measures included the CERAD neuropsychologic tests (Verbal Fluency, Boston Naming, MMSE, Word List Memory, Recall and Recognition), Logical

Memory and Digit Span subtests of the Wechsler Memory Scale - Revised, East Boston Memory Test, and modified versions of the Symbol Digit Modalities Test, Judgment of Line Orientation, Complex Ideational Material, Number Comparison, Digit Ordering, Standard Progressive Matrices, and National Adult Reading Test. A board-certified neuropsychologist, blinded to all clinical data except education, occupation, visual acuity, and information about hearing loss, cooperation, and effort, reviewed all test results, and rated the presence of impairment in five cognitive areas (orientation, attention, memory, language, and perception); an opinion regarding the probability of dementia and Alzheimer's disease was also rendered for cases with borderline dementia.

Clinical diagnoses were made by a board-certified neurologist with expertise in the evaluation of older persons after review of all clinical data and an examination of the participant. The neurologist reviewed a summary of the medical history, medication use, neurologic examination findings, and results of cognitive performance testing and the neuropsychologist's opinion of impairment. Each participant was evaluated in the subject's home, emphasizing findings deemed clinically relevant. The diagnosis of dementia and AD followed the recommendations of the joint working group of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS/ADRDA) (McKhann et al., 1984) and required loss of memory and other cognitive function by neuropsychological performance testing. Consensus criteria for the clinical classification of mild cognitive impairment has yet to be established. For the present study, this group included persons with impairment on neuropsychological testing of insufficient severity to warrant a diagnosis of dementia. Included in the present investigation are 33 subjects meeting the criteria either for AD (n = 11), mild cognitive impairment (MCI; n = 11), and those with no cognitive impairment (NCI; n = 11), who did not have a coexisting condition judged to be contributing to cognitive impairment. Of these, only 22 subjects (6 NCI, 7 MCI, and 9 AD) were suitable for stereologic evaluation due to loss of sections during processing. Information regarding these 22 cases is given in Table 1.

TABLE 1. Summary of Cases[1]

Case	Age (yr)	Gender	Education (yr)	ApoE	PMI (hr)	Pathologic Dx[a] CERAD	Pathologic Dx[a] Khach.	MMSE
NCI								
16	85	M	18	3,4	5.0	Normal	Not AD	28
18	92	M	20	3,3	11.0	Normal	Not AD	26
25	66	M	24	3,3	5.0	Normal	Not AD	29
27	82	M	16	3,3	3.8	Poss AD	Not AD	28
33	90	M	16	3,3	7.5	Poss AD	Not AD	27
41	81	F	16	3,4	3.9	Normal	Not AD	28
MCI								
11	84	M	21	3,4	16.0	Poss AD	AD	24
12	81	F	20	3,3	13.0	Normal	Not AD	27
13	81	F	16	4,4	8.0	Poss AD	Not AD	20
15	80	F	20	3,3	5.0	Poss AD	Not AD	26
17	80	M	21	3,3	4.5	Poss AD	Not AD	27
30	92	F	16	3,4	6.0	Poss AD	Not AD	28
37	82	F	18	3,4	14.0	Normal	Not AD	28
AD								
4	83	F	16	3,4	6.0	Def AD	AD	25
20	85	F	20	3,4	1.0	Def AD	AD	25
21	75	F	16	3,3	4.0	Prob AD	AD	27
28	93	M	6	3,3	3.5	Prob AD	AD	14
31	87	M	19	3,3	3.0	Poss AD	Not AD	24
43	87	M	8	3,3	15.0	Prob AD	AD	20
45	80	M	18	3,4	4.5	Def AD	AD	17
54	94	F	16	2,4	4.0	Prob AD	AD	28
56	77	M	19	3,4	4.8	Def AD	AD	29

[1]Pathologic diagnosis refers to autopsy findings using either CERAD criteria for normal, possible AD (Poss AD), probable AD (Prob AD), and definite AD (Def AD), or Khachaturian (Khach.) criteria for not AD and AD. PMI, postmortem interval; MMSE, Mini-Mental Status Examination score; NCI, no cognitive impairment; MCI, mild cognitive impairment; AD, Alzheimer's disease; ApoE, apolipoprotein E.

Brain tissue and pathology

After removal of the brain, one hemisphere of each brain was cut on a brain-slice apparatus into 1-cm-thick slabs, immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 48 hours at 4°C, and cryoprotected in 10% glycerol plus 2% dimethyl sulfoxide (DMSO) in 0.1 M phosphate buffer at 4°C for two days, followed by a solution of 20% glycerol plus 2% DMSO. Brain slabs containing the nucleus basalis of Meynert were cut into 18 series (720-µm interval) of adjacent 40-µm-thick sections on a freezing, sliding microtome and stored in cryoprotectant until processed. Select regions from the other hemisphere were paraffin embedded, cut, and stained with a modified Bielschowsky stain for neuropathologic diagnosis.

A pathologic diagnosis was made for all cases. The CERAD designations of "normal" (with respect to AD or other dementing processes), "possible" or "probable AD", and "definite AD" were based on semiquantitative estimation of neuritic plaque density, an age-related plaque score, and presence or absence of dementia (Mirra et al., 1991). The Khachaturian designations of "not AD" or "AD" were also made based on the presence of neurofibrillary tangles and a minimal number of neuritic plaques in three neocortical fields, as well as on age and clinical diagnosis of dementia (Khachaturian, 1985).

Immunocytochemistry

After several rinses in Tris-buffered saline (TBS) pH 7.1 to remove cryoprotectant, sections were incubated in a 0.1 M sodium periodate/TBS solution for 20 min to inhibit endogenous peroxidase activity. After three 10-minute rinses in TBS/0.25% Triton X-100 (TBS/TX) buffer, the sections were incubated in TBS/TX buffer containing 10% normal horse serum (NHS; ChAT) or normal goat serum (VAChT) for one hour. The sections were then rinsed twice in TBS/TX/3% serum. Sections were incubated with agitation overnight at room temperature in TBS/TX/3% serum with one of the following primary antibodies: polyclonal goat anti-human placental ChAT (1:1,000) or polyclonal rabbit anti-human VAChT C-terminus (1:5,000). Characterization of the ChAT (German et al., 1985) and VAChT (Schafer et al., 1995) antibodies has been described previously. After three rinses in TBS/3% serum, sections were incubated in either biotinylated horse anti-goat (ChAT) or biotinylated goat anti-rabbit (VAChT) secondary antibody (1:200; Vector) for one hour. Sections were subsequently rinsed three times in TBS and incubated in avidin-biotin-complex (ABC Elite; Vector) for one hour. Immunoreactivity was visualized by using a modification of the Hancock method (Hancock, 1982). The sections were rinsed three times with 0.2 M sodium acetate and 1.0 M imidazole buffer, pH 7.4. The chromagen solution contained 0.05% diaminobenzidine (DAB), 2.5% nickel II sulfate, and 0.005% H_2O_2, pH 7.2 in acetate/imidazole buffer. The reaction was terminated with three rinses in acetate/imidazole buffer. For light microscopic analysis, sections were mounted onto subbed slides, dehydrated in alcohols, defatted in Histo-

clear (National Diagnostics) and cover-slipped. Some sections were further processed for electron microscopy to evaluate the penetration of immunostaining. Sections were fixed with 1% osmium tetroxide, dehydrated in ethanol, and flat embedded in Epon. Strips containing the nucleus basalis were microdissected and reembedded. Sections (50–60 nm) were cut perpendicularly to the original section by using an ultramicrotome, and analyzed by electron microscopy. Light microscopic images were captured electronically by using a high resolution Sony CCD camera and MCID software (version 3.0, rev. 1.4; Imaging Research). Electron micrograph negatives were scanned into a MacIntosh computer by using SprintScan software (Polaroid). Images were composed by using Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA), with brightness and contrast optimized for each photograph.

Definition of nucleus basalis of Meynert

The ChAT and VAChT immunopositive neurons were counted along the entire rostrocaudal extent of the nucleus basalis of Meynert (Ch4) as previously defined (Mesulam and Geula, 1988). The rostral delineation between the vertical limb of the diagonal band (Ch2) and the anteromedial division of the nucleus basalis (Ch4am) was determined by the location of a group of large multipolar immunopositive neurons under the anterior commissure. Caudally, the posterior nucleus basalis neurons (Ch4p) were delineated from neurons of the putamen based on morphologic criteria, including size. Because there are very few neurons in the horizontal limb of the diagonal band (Ch3) in the human (Mesulam and Geula, 1988), this group was included with the neuron counts of the nucleus basalis. Other scattered CBF, which are part of the nucleus basalis, were also included such as the interstitial neurons located within the internal capsule, medullary laminae of the globus pallidus, ansa peduncularis, and ansa lenticularis.

Stereologic analysis of total ChAT and VAChT neuron number

The total number of ChAT and VAChT immunopositive neurons within the nucleus basalis was determined by using the optical fractionator (Gundersen, 1986; West et al., 1991). The optical fractionator is a design-based stereologic method for estimating total number by using a stack of optical sections to determine the number of neurons in a known fraction of a defined reference space. Briefly, the optical fractionator system consisted of a computer assisted image analysis system, including a Nikon Microphot-SA microscope hard-coupled to an ASI computer-controlled x-y-z motorized stage, a high-sensitivity Sony CCD video camera system, and a Macintosh Power PC computer. All analyses were performed by using Stereologer software (Systems Planning and Analysis, Inc.) by an observer blinded to both the clinical diagnosis and immunocytochemical stain for each series. Before each series of measurements, the instrument was calibrated. The fractionator sampling scheme used was adjusted after performing a pilot study on four control and four AD brains immunostained for ChAT. Beginning at a random starting position, immunopositive neurons were counted on all sections containing the nucleus basalis from a 1 in 18 series. The nucleus basalis was outlined at low magnification (1× objective, 29× final magnification on the monitor). Within this reference space, dissectors were placed at 1,000-μm steps along the x and y axis from a random start. Immunopositive neurons were counted in a 40,000 μm^2 counting frame according to dissector counting rules (West et al., 1991; West, 1993) by using a 20× objective (569× final magnification on the monitor). The average thickness of the sections was measured at 14.5 μm. However, neurons were only counted within a 10 μm height of tissue, with guard heights of 2.5 μm at the top and 2.0 μm at the bottom of the sections. Antibody penetration was complete as assessed by electron microscopy as shown in the Results section. The total number of each type of labeled perikarya (N) within the nucleus basalis was estimated by using the following formula:

$$N = \Sigma Q^- \cdot 1/ssf \cdot 1/asf \cdot t/h$$

where ΣQ^- is the total number of neurons actually counted in the dissectors that fell within the sectional profiles of the sampled sections (Gundersen, 1986; West et al., 1991). The fractions used to calculate the total number of objects were (1) section sampling fraction (ssf) = 1/18; (2) area sampling fraction (asf) = 40,000 $\mu m^2/(1{,}000\ \mu m)^2$; and (3) thickness of section (t) = 14.5 μm and height (h) =10 μm. By using this systematic sampling scheme from random starting positions, a minimum of 100 neurons were actually counted for each series as suggested for reliable stereologic estimates (West et al., 1991).

The coefficient of error (CE), which represents methodological error, was calculated individually for each series by using the formula: CE = the square root of the number of neurons sampled per case divided by the number of neurons sampled. This error ranged from 5 to 10% across cases. The quadratic approximation of CE was not used because more than 10 sections were evaluated for each case. The CEs of individual cases were then averaged by clinical diagnosis and by cholinergic marker. The total variation (CV) was calculated by dividing the standard deviation by the mean total neuron counts for "n" individuals for each group. The biological variation (BV) was then calculated by using the formula $BV^2 = CV^2 - CE^2$ as described (West et al., 1991; West, 1993).

Statistical analysis

The total number of ChAT and VAChT immunopositive neurons were compared among the three clinically defined groups by one-way analysis of variance (ANOVA). These neuron counts were subsequently compared with a number of variables and analyzed as follows. MMSE scores, education, age at death, and postmortem intervals were compared with neurons counts by correlation analysis. The total number of ChAT or VAChT immunopositive neurons from all clinically defined groups were compared with gender, Khachaturian pathologic diagnosis, and ApoE e4 status (possessing an ApoE e4 allele or not) by means of unpaired t-tests. The CERAD pathologic diagnosis was compared with neuron counts by ANOVA. The significance for all statistical tests was set to $P < 0.05$ (two-tailed).

RESULTS

General characteristics of immunostaining for ChAT and VAChT

Tissue stained for ChAT and VAChT by using nickel intensification yielded dark blue reaction product located within the cytoplasm (Figs. 1, 2). These neurons typically

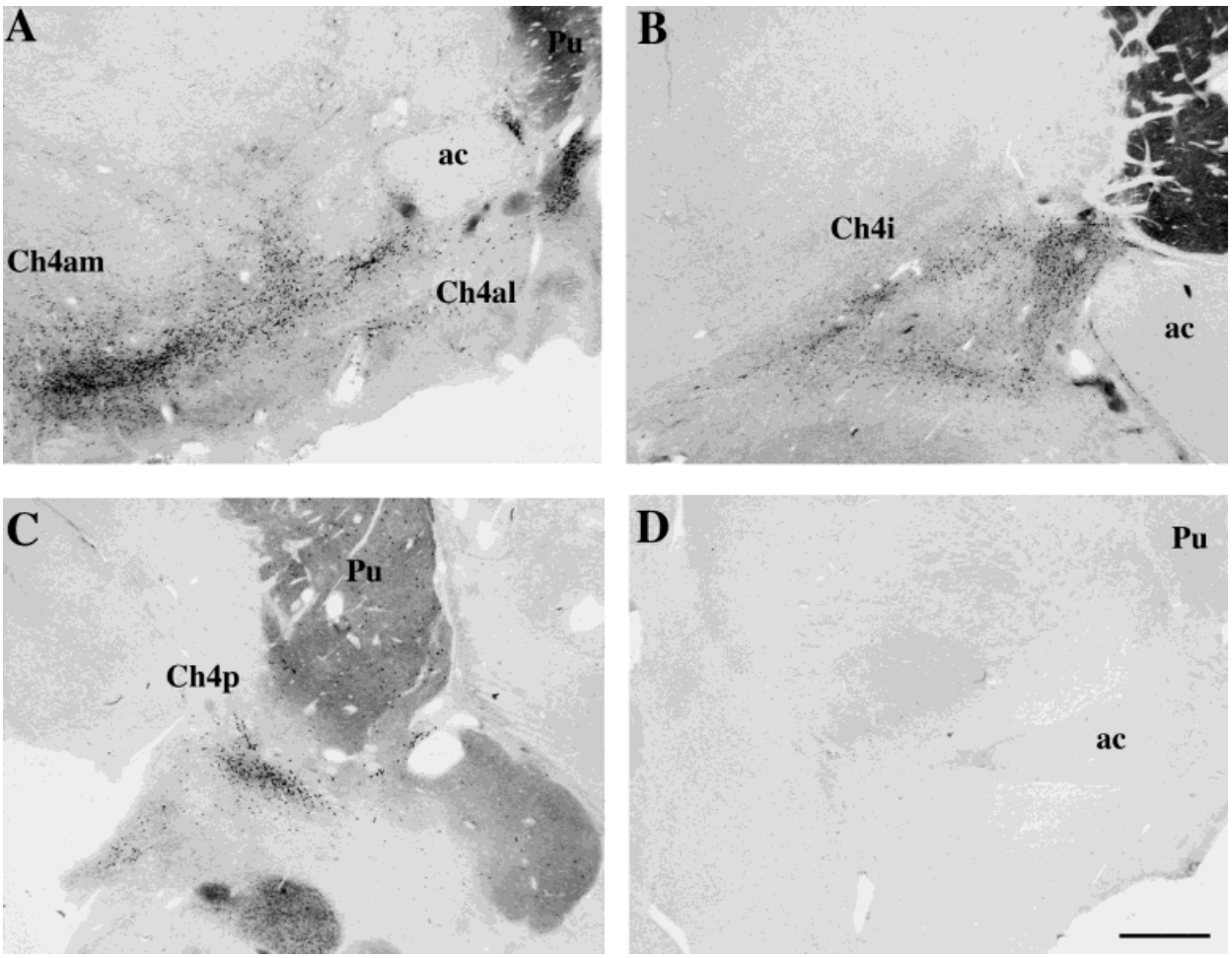

Fig. 1. Immunocytochemical localization of vesicular acetylcholine transporter-immunoreactive neurons immunoreactivity in the human nucleus basalis of Meynert. Shown are representative stained sections from rostral (**A**), intermediate (**B**), and caudal (**C**) levels of the basal forebrain from individuals with no cognitive impairment. The subdivisions of the nucleus correspond to those described previously by using choline acetyltransferase-immunoreactive neurons to mark the cholinergic neurons (Mesulam and Geula, 1988). Also note the high density of immunoreactivity in the putamen and amygdala. **D:** Staining was abolished when primary antibody was omitted. ac, anterior commissure; Ch4am, anteromedial cell group; Ch4al, anterolateral cell group, Ch4i, intermediate cell group; Ch4p, posterior cell group; Pu, putamen. Scale bar = 2 mm.

possessed one to four proximal dendrites, which were also filled with reaction product. Extensive fiber labeling was also observed by using both markers. In several, but not all, of the confirmed AD cases, cellular debris and dystrophic neurites were present and were ChAT or VAChT immunopositive (Fig. 2C,D). There was also punctate labeling, seen more prominently with VAChT throughout the nucleus basalis, which was often in close association with immunopositive neurons. In the few AD cases in which neuron number was noticeably decreased, there was no apparent change in the density of VAChT-immunostained puncta.

Distribution of ChAT and VAChT immunoreactive neurons in control cases

ChAT and VAChT immunopositive neurons formed a continuum of labeled perikarya throughout the subgroups of nucleus basalis of Meynert in the NCI cases (Fig. 1). In fact, the distribution of VAChT-immunopositive neurons closely matched that of ChAT-stained perikarya throughout the nucleus basalis as previously described (Mesulam and Geula, 1988). In anterior sections, VAChT immunopositive neurons were found in a compact region below the crossing of the anterior commissure, corresponding to the anteromedial division of the nucleus basalis (Ch4am). At the emergence of the anterior commissure, this cell group merged with the anterolateral subfield (Ch4al: Fig. 1A). At the level of ansa peduncularis, VAChT-immunopositive neurons were seen occupying the intermediodorsal (Ch4id) and intermedioventral (Ch4iv) subdivisions of the nucleus basalis complex (Fig. 1B). Further caudally, VAChT-immunopositive cell bodies were nestled between the most ventral aspects of the putamen and external segment of the globus pallidus comprising the posterior subfield (Ch4p) of the nucleus basalis (Fig. 1C). Additionally, there were interstitial nucleus basalis VAChT-immunopositive neurons scattered within the internal capsule, medullary laminae of the globus pallidus, ansa peduncularis, and ansa lenticularis. Large VAChT-immunopositive neurons, characteristic of cholinergic interneurons, were found

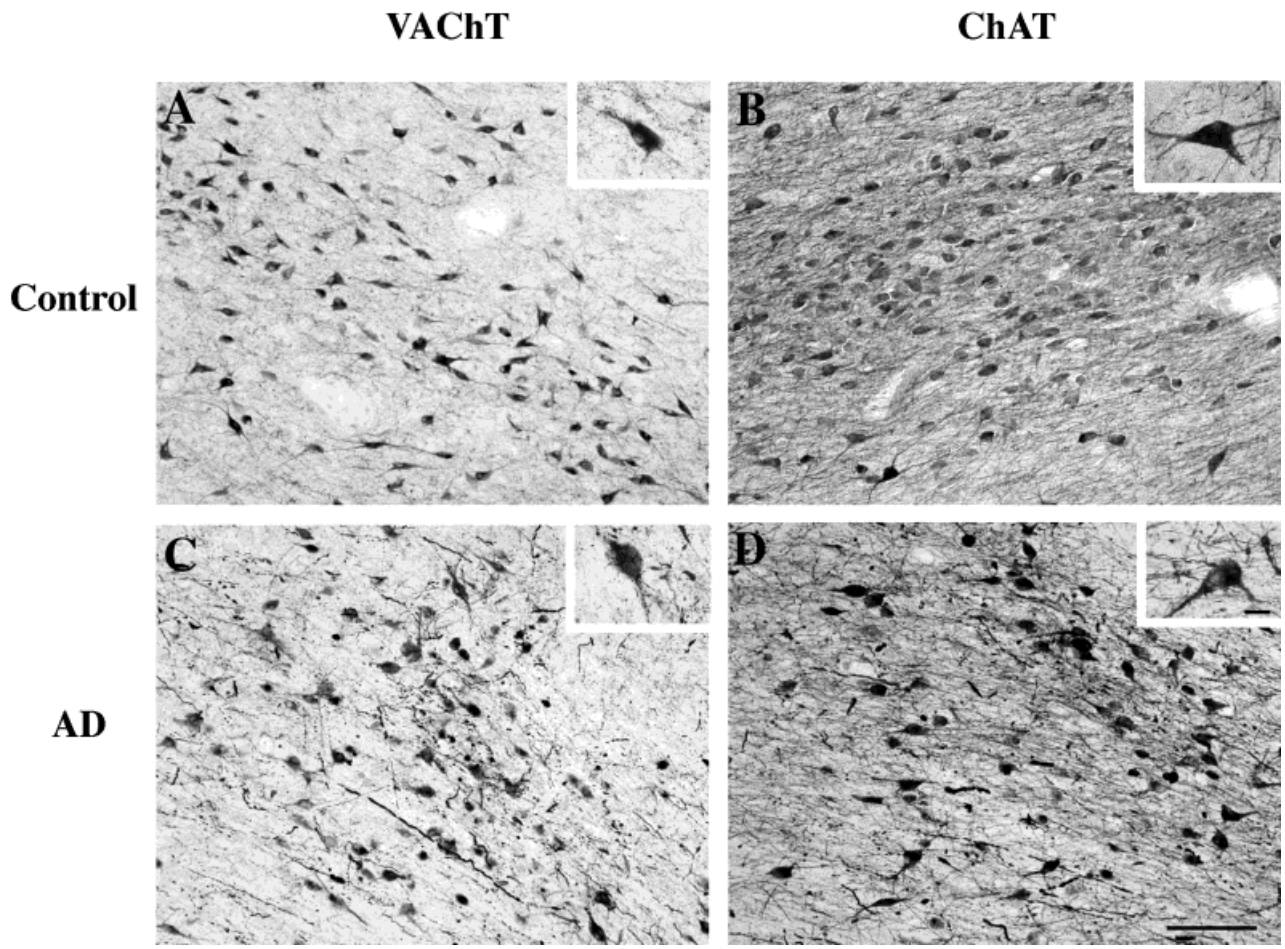

Fig. 2. Vesicular acetylcholine transporter– (VAChT) and choline acetyltransferase– (ChAT) immunoreactive neurons in the nucleus basalis of Meynert in Alzheimer's disease (AD) and no cognitive impairment. VAChT (**A,C**) and ChAT (**B,D**) immunoreactivity were generally similar, as shown in sections from control brains with no cognitive impairment (A,B) and in an AD case (C,D). In this AD case, there were frequent dystrophic neurons, swollen processes, and cellular debris seen with both cholinergic markers. Scale bar = 200 μm in D (applies to A–D), 20 μm in insets.

throughout the caudate and putamen, which also contained dense punctate labeling (Fig. 1). In a few cases in which the temporal lobe was attached to the basal forebrain blocks, there was robust staining of VAChT- and ChAT-immunopositive fibers in the basolateral amygdala and adjacent cortex (data not shown). No cortical cell bodies were observed with either marker. Tissue sections processed with the omission of primary antibody failed to reveal any immunoreactive profiles (Fig. 1D) in brains from NCI or cognitively impaired individuals.

Stereologic analysis of total ChAT and VAChT neurons

In the optical fractionator method as used here, object counts were performed within the depth of the immunostained sections. An important assumption with stereology is that objects have an equal opportunity to be counted at varying depths in the tissue. It is well known from immunoelectron microscopic studies that antibody penetration is often restricted to a few microns from the tissue surfaces. However, by design such studies typically use tissue processed in a manner to preserve ultrastructural morphology (e.g., perfusion fixation and minimal detergent exposure). In contrast, in the present study the brain tissue was processed after postmortem delays with immersion fixation, and immunostaining was performed with high detergent concentrations to permeabilize tissue and increase antibody penetration. By light microscopic analysis with controlled steppage of focal planes through the tissue, penetration appeared complete. For more detailed analysis, immunostained tissue was further examined by electron microscopy after thin sectioning the tissue perpendicular to the original plane of section. As shown in Figure 3, immunoreactivity for both markers extended throughout the depth of the tissue.

Having established that tissue penetration was complete and after optimizing the sampling scheme with a pilot study (see the Materials and Methods section), the total number of ChAT- and VAChT-immunopositive neurons in the nucleus basalis of Meynert was estimated in six NCI cases by using the optical fractionator as described in the Materials and Methods section. The average number of ChAT immunopositive neurons was 210,540 compared with 174,000 VAChT immunopositive neurons (Table 2). There was no significant difference between the total number of ChAT- and VAChT-immunopositive neurons by

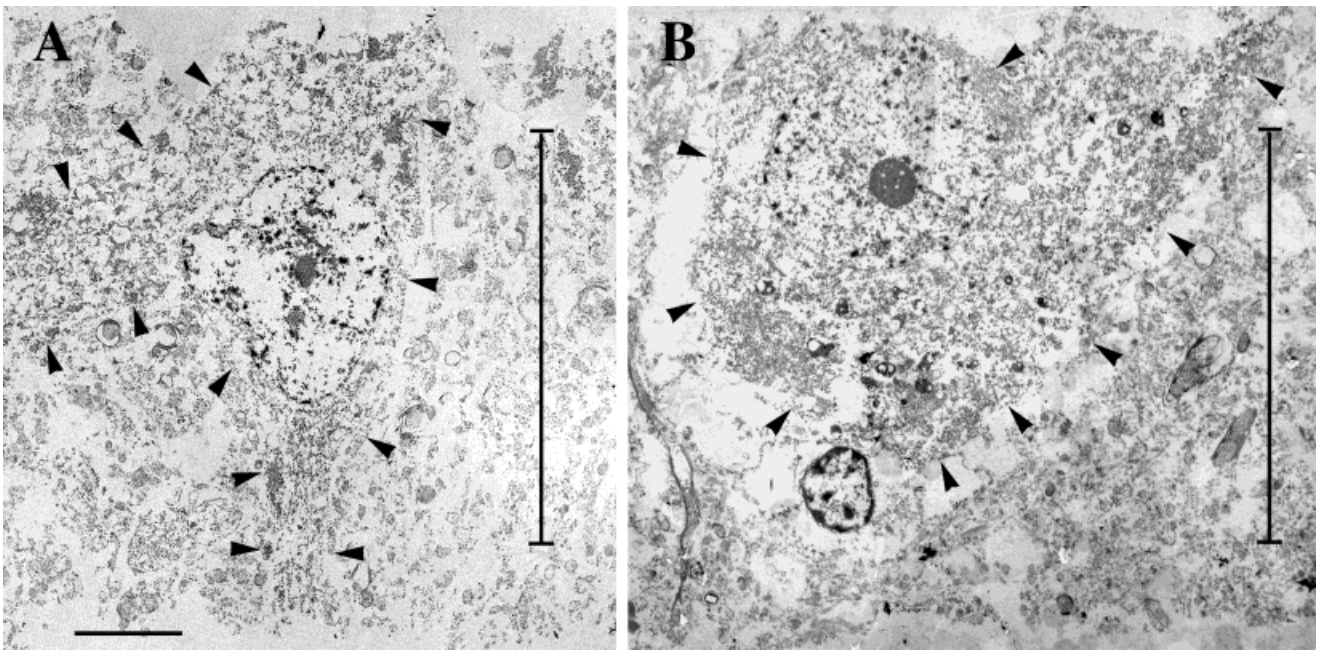

Fig. 3. Electron microscopic analysis of choline acetyltransferase-immunoreactive neurons (ChAT) (**A**) and vesicular acetylcholine transporter-immunoreactive neurons (VAChT) (**B**) immunoreactivity in the nucleus basalis. To assess the tissue penetration of immunostaining, thin sections were processed perpendicular to the original section as described in the methods. The photomicrographs depict ChAT-immunoreactive (A) and VAChT-immunoreactive (B) neurons (outlined by arrowheads) that nearly span the entire thickness of the tissue. Note that immunoreactivity is present throughout the depth of the sections. As expected, the ultrastructural preservation is poor because of the manner in which the tissue was obtained and processed. The vertical bar approximates the portion of the section depth that was used for stereologic analysis, reflecting guard heights at the top and bottom of the sections. Scale bar = 10 μm in A (applies to A,B).

TABLE 2. ChAT- and VAChT-Immunoreactive Neurons in the Nucleus Basalis of Meynert[1]

Cases	ChAT (mean ± standard deviation)	VAChT (mean ± standard deviation)
NCI	210,540 ± 15,240 (n = 6)	174,000 ± 12,773 (n = 6)
MCI	167,879 ± 17,903 (n = 7)	192,637 ± 34,737 (n = 5)
AD	155,585 ± 17,949 (n = 9)	149,423 ± 17,615 (n = 9)

[1]ChAT, choline acetyltransferase–immunoreactive neurons; VAChT, vesicular acetylcholine transporter–immunoreactive neurons; NCI, no cognitive impairment; MCI, mild cognitive impairment; AD, Alzheimer's disease.

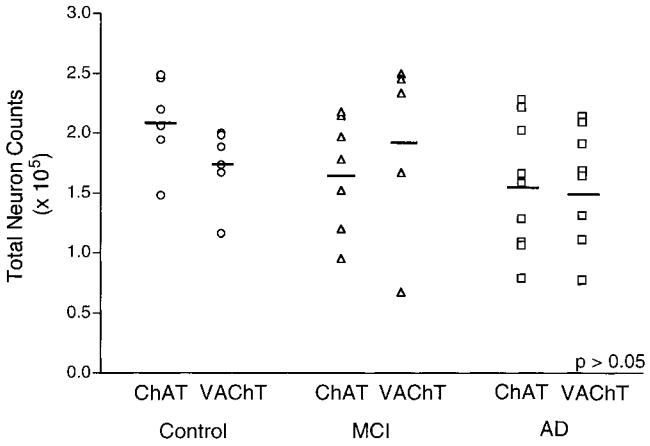

Fig. 4. Summary of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) total neuron counts. Circles represent no cognitive impairment (NCI) cases, triangles represent mild cognitive impairment (MCI) cases, and squares represent Alzheimer's disease (AD) cases. Total neuron counts were estimated in the same NCI, MCI, and AD cases for ChAT and VAChT. There was no significant difference between the number of ChAT- and VAChT-immunopositive neurons for any of the three clinically diagnosed groups. Bars represent the mean.

t-test in the NCI group ($t = 1.84$, $P = 0.1$; Fig. 3). The calculated biological variation for the NCI group was approximately 17%.

The optical fractionator method was also used to calculate the total number of ChAT- and VAChT-immunopositive neurons for clinically defined MCI (n = 7, ChAT; n = 5, VAChT) and AD (n = 9, ChAT and VAChT) cases. In the MCI group, the average number of ChAT-immunopositive neurons was 167,879 compared with 192,637 VAChT-immunopositive neurons. In the AD group, the average number of neurons immunoreactive for ChAT was 155,585 compared with 149,423 VAChT containing perikarya. There was no significant difference between the number of ChAT- and VAChT-immunopositive neurons for either the MCI ($t = -0.069$, $P = 0.51$) or AD ($t = 0.245$, $P = 0.81$) clinically defined groups by t-test (Fig. 4). Moreover, there was no significant difference in the average total number of ChAT ($F = 2.47$, $P = 0.11$) or VAChT immunopositive neurons ($F = 1.05$, $P = 0.37$) comparing across the NCI, MCI, and AD groups by ANOVA. Combining all groups, there was a significant correlation between the number of ChAT- and VAChT-immunopositive neurons ($r = 0.81$, $P < 0.0001$; Fig. 5). The calculated biological variation was approximately 35% in both the MCI and AD groups.

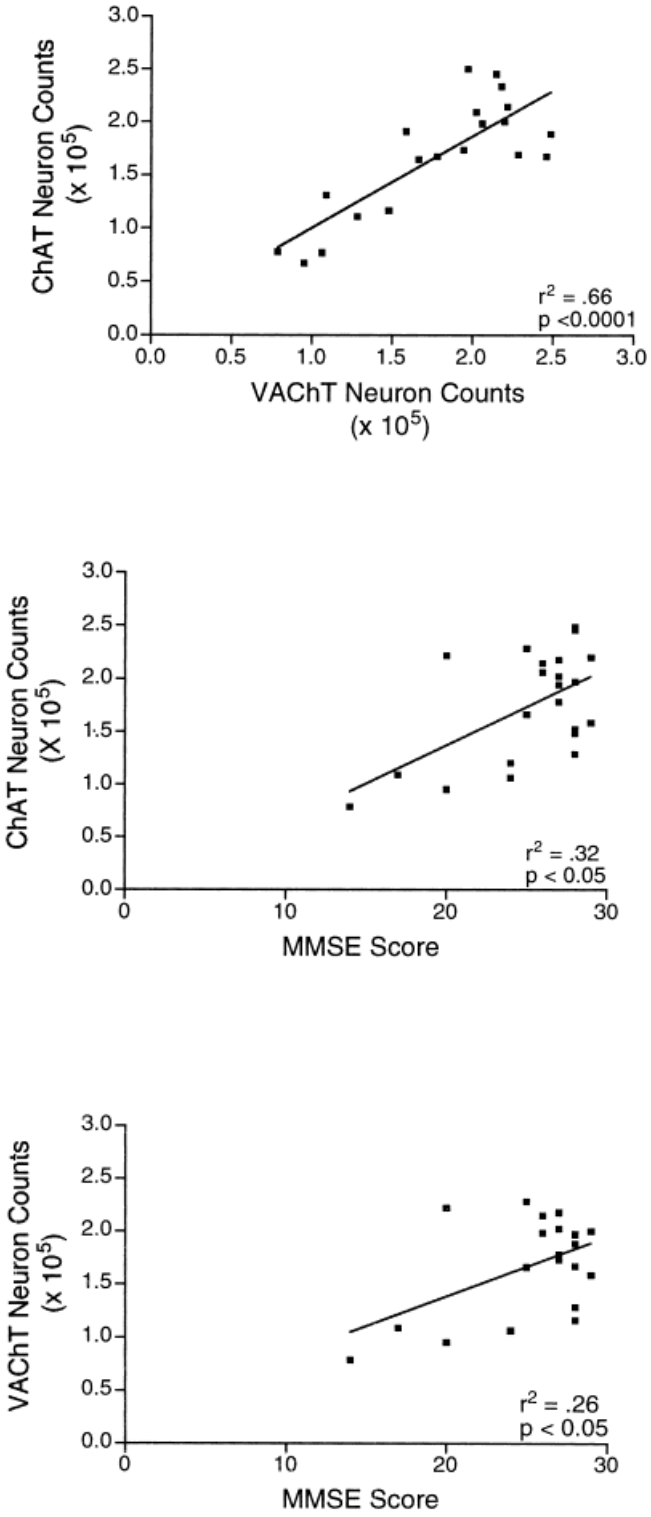

Fig. 5. Correlation of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) total neuron counts with each other and Mini-Mental Status Examination (MMSE) scores.

The data were reanalyzed by using pathologic criteria to distinguish AD and non-AD cases (Table 1). Because there has been some controversy regarding the best pathologic definition of AD, in particular to objectively distinguish AD from normal age-related changes (Mirra et al., 1993), two standard pathologic criteria were applied to the 22 cases used for stereology in the present study. When grouped by CERAD pathologic diagnosis, there was no significant difference among the groups (normal, possible or probable AD, definite AD) for the number of ChAT ($F = 0.93$, $P = 0.414$) or VAChT-immunopositive neurons ($F = 0.41$, $P = 0.670$) compared by ANOVA. When grouped according to Khachaturian criteria, there was no significant difference between the AD and non-AD groups for the number of ChAT- ($t = 1.63$, $P = 0.118$) or VAChT-immunopositive neurons ($t = 0.594$, $P = 0.56$) compared by t-test.

Correlation between ChAT and VAChT immunoreactive neurons with other variables

The total number of ChAT and VAChT immunopositive neurons was compared with several demographic variables (Table 1). Each variable was correlated with neuron counts by combining data from all cases as well as by analysis within each clinically designated group. Combining all clinically diagnosed groups revealed a significant correlation between the score on the MMSE, a brief cognitive test rating dementia severity (Folstein et al., 1975), and the number of ChAT- ($r = 0.56$, $P < 0.01$) and VAChT- ($r = 0.6$, $P < 0.01$) immunopositive neurons (Fig. 5). There was a significant correlation between the MMSE score and the number of ChAT- ($r = 0.76$, $P < 0.05$) and VAChT- ($r = 0.90$, $P < 0.05$) immunostained neurons for the MCI but not the NCI or AD cases.

The ApoE 4 allele has been shown to correlate strongly with several variables in AD, including cortical ChAT activity (Poirier, 1994; Soininen et al., 1995). Interestingly, possession of an ApoE 4 allele may decrease responsiveness to acetylcholinesterase inhibitor therapy due to a more severe cholinergic deficiency (Poirier et al., 1995). In the current study, there was no significant association between possession of an ApoE 4 allele and the number of ChAT- or VAChT-immunopositive neurons when the three clinically diagnosed groups were combined or evaluated separately. Although almost half of the cases examined possessed at least one ApoE 4 allele, the present sample population was small and the power to detect such an association was low.

Combining all clinically diagnosed groups there was no significant difference for age at time of death, gender, education, postmortem interval, and number of ChAT- or VAChT-immunopositive neurons. Within each diagnosis, there was also no significant correlation with either number of ChAT or VAChT immunopositive neurons.

DISCUSSION

The present study is the first immunohistochemical investigation comparing the distribution and total number of neurons containing ChAT and VAChT, two protein products of a unique cholinergic gene locus, within the nucleus basalis in humans with varying levels of cognitive function. The individuals studied were examined 12 months proximate to death and based on clinical evaluation were grouped into those with no cognitive impairment, mild cognitive impairment, and AD. It is important to emphasize that the AD cases were very mild. This investigation revealed that VAChT, like ChAT, is an excellent marker for

cholinergic neurons and fibers within the human nucleus basalis of Meynert. Stereologic counts of ChAT- and VAChT-stained neurons demonstrated that the total number of cholinergic neurons in the nucleus basalis was between 190,000 and 200,000 per hemisphere. Interestingly, the analysis did not reveal a significant decrease in the number of cholinergic neurons in MCI or AD compared with cognitively intact individuals, due in part to the large biological variation observed even in the latter group. Moreover, in contrast to recent pathologic studies suggesting disparate changes in the levels of these markers within cholinergic terminal fields in AD, there was no significant difference between the number of ChAT- and VAChT-immunopositive neurons within the nucleus basalis.

VAChT is a marker of cholinergic neurons and terminals

The distribution of VAChT-containing neurons within the nucleus basalis from the NCI group was similar to the pattern seen in studies that used antibodies against ChAT or the low-affinity nerve growth factor receptor, another excellent marker for CBF neurons in the human brain (Mesulam and Geula, 1988; Mufson et al., 1989a). Punctate VAChT labeling was widely distributed throughout the nucleus basalis and was often found in close association with immunopositive neurons. This staining likely represents cholinergic terminals (Gilmor et al., 1996), originating either from collaterals of local cholinergic nucleus basalis neurons or from cholinergic neurons elsewhere in the brain.

Stereologic counts of VAChT and ChAT immunopositive neurons in the nucleus basalis

Although stereologic techniques have been promoted over traditional counting methods, they are not without considerable controversy (Coggeshall and Lekan, 1996; Saper, 1996; Guillery and Herrup, 1997; Saper, 1997). Proponents of stereology have described the advantages of stereologic methods in great detail (Gundersen, 1986; West, 1993). Briefly, the advantages of stereologic counting techniques are (1) no assumptions are made regarding the shape, size, or orientation of the objects being counted; (2) only a small number of objects are counted to obtain reliable estimates of total object number; (3) stereology allows determination of total object number, not object density. This is particularly important when gross tissue shrinkage may occur, as in AD. This change in overall tissue volume could seriously affect density estimates but is accounted for with stereologic estimates of total object number.

Estimates of the total number of cholinergic neurons within the nucleus basalis of Meynert have ranged from 100,000 to 400,000 by using tissue sections stained for Nissl substance and traditional counting methods (McGeer et al., 1984; Arendt et al., 1985; Lehericy et al., 1993). The present results estimate the number of cholinergic neurons to be 190,000–200,000 per hemisphere, which are in close agreement with studies that used stereologic methods or cholinergic specific markers. Vogels et al. (1990) evaluated Nissl-stained sections from control cases by using a similar optical fractionator scheme and a neuron diameter of greater than 37.5 μm to define cholinergic neurons. The average total number of presumptive cholinergic neurons was 179,000 and 195,000 for the right and left hemispheres, respectively. By using immunocytochemistry for the nerve growth factor receptor as a marker for ChAT containing neurons (Mufson et al., 1989a), the total number of cholinergic neurons was estimated to be 190,000 per hemisphere (Mufson et al., 1989b). Considering that we found the biological variation in the NCI cases to be 17%, the number of cholinergic neurons in one hemisphere of the nucleus basalis of Meynert could range from 170,000 to 220,000 in any healthy aged individual.

Loss of cholinergic neurons in AD

Despite the frequency of published reports indicating severely decreased numbers of basal forebrain neurons in AD (for review see Vogels et al., 1990), only one previous stereologic study addressed this issue. Vogels and coworkers (Vogels et al., 1990) reported a significant 20% decrease in the total number of Nissl-stained neurons within the nucleus basalis, regardless of perikaryal size in AD. When the total number of neurons was examined by size category, there was a 68–75% decrease of large neurons, but in contrast, a 40–80% increase in smaller neurons. Although the large neurons were presumably cholinergic, the basal forebrain is admixed with neurons containing a variety of neurotransmitters (Kohler et al., 1984; Mufson et al., 1988; Geula et al., 1993). Our study used two specific markers, ChAT and VAChT, to identify cholinergic neurons together with stereologic counting techniques. In our AD cases, there was an average decrease of 25% in the total number of ChAT neurons and 17% in VAChT neurons that approached, but did not reach, statistical significance. Given the biological variation of the NCI cases, an increase in the sample size used in the present study may result in a statistical difference between groups. Nonetheless, both the current and previous stereologic studies suggest that the actual loss of cholinergic neurons is smaller than many studies that used assumption-based methods suggested. Although the size of ChAT and VAChT neurons was not examined in the current study, the data are consistent with the hypothesis that there is a relative preservation of cholinergic neurons, albeit in a shrunken state, as has been proposed previously using either assumption-based (Perry et al., 1982; Pearson et al., 1983; Allen et al., 1988) or design-based stereologic quantitative methods (Vogels et al., 1990).

There are several possible explanations for the surprisingly small change, if any, in numbers of cholinergic neurons in AD. These results likely reflect the early stage of dementia for the cases examined as the MMSE score averaged about 23 of 30. Another factor influencing cholinergic vulnerability is age of onset. Although the actual age of onset of symptoms was not available for all the AD cases examined, they would qualify as late-onset AD based on the average age at the time of death (84.6 years). In fact, previous studies with early-onset AD (age at time of death in the late sixties and early seventies) have shown a greater reduction of cholinergic neurons within the nucleus basalis as compared with late-onset cases (Tagliavini and Pilleri, 1983; Allen et al., 1988). Studies of ChAT activity in the cortex have shown that ChAT activity is less severely decreased with late-onset and that these decreases are generally limited to the temporal cortex (Bird et al., 1983; Rossor et al., 1984; Perry et al., 1992; Sparks et al., 1992). Moreover, in vivo VAChT binding also appeared to be less severe and limited to the temporal cortex with late-onset (Kuhl et al., 1996). Therefore, the nonsignificant decrease

in the number of ChAT- and VAChT-immunopositive neurons in the nucleus basalis in the current study may also, in part, reflect the late onset of AD.

The results of the present investigation suggest that there is only a limited loss of cholinergic neurons in individuals with mild AD. In contrast, previous quantitative studies of cholinergic neuron loss within the basal forebrain have used postmortem tissue from severely demented individuals (Whitehouse et al., 1981; Wilcock et al., 1982; Tagliavini and Pilleri, 1983; McGeer et al., 1984; Arendt et al., 1985; Mufson et al., 1989b; Lehericy et al., 1993). The gravity of the dementia in these cases indicates that the progression of the disease was far advanced. Because no difference in number of cholinergic perikarya was found between either the MCI or mild AD compared with the NCI cases, this argues that basal forebrain neuron loss does not occur in the early stages of the disease process. This result is in contrast to findings in cholinergic terminal fields, where cortical ChAT activity decreases of 60% have been observed in biopsies from individuals with definite AD (Bowen et al., 1981, 1982, 1983; Sims et al., 1983; DeKosky and Scheff, 1990; DeKosky et al., 1992). There was a positive correlation between cholinergic neuron counts (by using either VAChT or ChAT) and cognitive function as has been observed previously (Lehericy et al., 1993), similar to the correlation reported between cortical ChAT activity and dementia severity (Perry et al., 1978, 1985; Wilcock et al., 1982; Ruberg et al., 1990; DeKosky et al., 1992; Bierer et al., 1995). However, this correlation was relatively weak and was influenced largely by a few more advanced cases. Although the precise role that dysfunction of the cholinergic basal forebrain system plays in the symptoms of dementia remains to be determined, our findings suggest that death of cholinergic neurons is not a necessary prerequisite for the onset of dementia. Future studies of ChAT and VAChT levels in cortex from the same cases with basal forebrain cell counts will be necessary to draw any firm conclusions about the relationship between the cholinergic terminals and their cells of origin.

Coordinate expression of ChAT and VAChT in the nucleus basalis of Meynert

The ChAT and VAChT neuron counts in our study support coordinate as opposed to independent expression of these proteins in the perikarya of cholinergic neurons in the nucleus basalis in AD. Before these findings, two lines of evidence suggested that these two cholinergic markers were expressed independently. First, binding assays in the frontal or temporal cortex by using the VAChT antagonist vesamicol demonstrated a nonsignificant decrease or even an increase in VAChT levels in AD (Kish et al., 1990; Ruberg et al., 1990). Recent studies that used more specific vesamicol analogs in vivo and in postmortem tissue did show significant, albeit less severe, decreases in cortical VAChT levels (Kuhl et al., 1996; Efange et al., 1997) than have been consistently shown for ChAT activity. If these two cholinergic-specific proteins were differentially expressed in cholinergic axon terminals, then one would expect to find independent expression of protein levels in the perikarya of these neurons. Second, the genes for ChAT and VAChT have been cloned in *Caenorhabditis elegans*, *Drosophila*, two *Torpedo* species, mouse, rat, and human (Eiden, 1998). In all of these species, the VAChT gene is nestled within a five prime intron of the ChAT gene with regulatory elements and promoters present to support either coordinate or independent gene expression. In fact, mRNA levels have been found to be independently expressed in response to several *trans* acting transcription factors (Berrard et al., 1995; Berse and Blusztajn, 1995) and during development (Holler et al., 1996; Tian et al., 1996), suggesting that the two gene products are independently regulated under certain conditions. However, the present study suggests that the two cholinergic genes are coregulated in AD. We recently have observed similar findings in studies that used experimental cholinergic lesions in the rat (Gilmor et al., 1998). Nonetheless, studies of ChAT and VAChT mRNA would be necessary to clarify whether there is transcriptional coregulation. There are several other possible explanations for the discrepancy between the coordinate vs. independent changes in levels of the two cholinergic markers in basal forebrain neurons and terminal fields, respectively. If the levels of ChAT activity are significantly lower than those of VAChT in the terminal fields, this suggests a potential problem with the ChAT enzyme and not necessarily the level of protein actually present. Alternatively, the disease process may perturb the transport of soluble proteins such as ChAT differently than for membrane-bound proteins such as VAChT and HAChT (Perry et al., 1982; Etienne et al., 1986).

SUMMARY

The current results indicate that cholinergic neurons in the nucleus basalis of Meynert are mostly preserved and coordinately express ChAT and VAChT in the early stages of AD. Nonetheless, well-established decreases in ChAT activity in cholinergic terminal fields, together with demonstrated benefits of cholinergic therapies (Farlow et al., 1992; Bodick, 1997) suggests these neurons have impaired function. An important therapeutic implication of these findings is that survival of the basal forebrain cholinergic neurons in early stages of the disease provides an opportunity for interventions aimed at restoring function.

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