Morphometric Study on the Ch4 of the Nucleus Basalis of Meynert in Alzheimer's Disease

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ABSTRACT

Ch4 neurons were studied morphometrically in three normal controls and three patients with Alzheimer's disease (AD). Two series of preparations stained with cresyl violet and with the indirect immunoperoxidase method using a monoclonal antibody to acetylcholinesterase (AChE) counterstained with cresyl violet were morphometrically analyzed. The cholinergic neurons were identified as positive reaction products of AChE in the perikarya. The cross-sectional area of all the Ch4 neurons with clearly visible nucleoli in one preparation was measured using a computer imaging system. Evaluation of these data provided a mean value of the cross-sectional neuronal area (CNA), number of neurons, standard deviation of the CNA and coefficient of variation of the cholinergic and noncholinergic neurons. The cholinergic neurons were decreased in number but showed hypertrophy in the early stage of AD, and they gradually became atrophic in the advanced stage. The noncholinergic neurons showed only

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atrophy without definite neuronal cell depletion. These findings indicate that cholinergic neurons have a plasticity and remodeling property against the AD process and suggest that hypertrophy of the cholinergic neurons in the Ch4 is the result of the effect of neurotrophic factors.

Index Entries: Immunocytochemistry; morphometry; nucleus basalis of Meynert; acetylcholinesterase; Alzheimer's disease; cholinergic neurons.

INTRODUCTION

The central cholinergic system consists of six neuronal cell subpopulations comprising Ch1-6 (Mesulam et al., 1983a, 1983b). The nucleus basalis of Meynert (nbM) in the substantia innominata, designated as Ch4, is a major source of the cholinergic projection to the cerebral cortex (Mesulam et al., 1983a). The nbM is usually affected in Alzheimer's disease (AD) resulting in a hypocholinergic condition in the central nervous system, which is associated with mnemonic dysfunction and dementia (Whitehouse et al., 1982). The vulnerability of this nucleus in AD has been ascertained by immunocytochemical procedures, and various studies using morphometric analysis have reported loss and atrophy of the neurons in this nucleus (Allen et al., 1988; Chan-Palay, 1988; Kobayashi et al., 1990; Nakano and Hirano, 1985; Whitehouse et al., 1982). Recent studies have reported that nbM degeneration in AD is associated with hyperinnervation of the galanin-positive nerve cell process or a decrease in the number of neurons with galanin-like immunoreactivity (Chan-Palay, 1988; Vogels et al., 1989). The immunohistopathological observation by Chan-Palay (1988) raised the possibility that the cholinergic neurons in the Ch4 may be enlarged in AD and in Parkinson-dementia complex. Experimental neuropathological studies have demonstrated the unilateral lesions in the neocortex and nucleus basalis lead to contralateral neuronal cell hypertrophy in the nbM (Pearson et al., 1987). It is likely that neurons in the nbM have a plasticity and remodeling property against lesions of a certain cerebral region that affects the nerve endings of the cholinergic neurons. Furthermore, several studies on nerve growth factor (NGF) have found a selective neurotrophic action to the central cholinergic neurons (Gnahn et al., 1983; Hefti et al. 1985; Martinez et al., 1987; Rieger et al., 1980). Therefore, the neuronal cell size in the Ch4 may vary, probably owing to neurotrophic factors such as NGF. However, morphometric studies on the cholinergic neurons using immunocytochemical procedures have evaluated only neuronal cell counts (Nagai et al., 1983), and precise data on the morphometrical differences between cholinergic and noncholinergic neurons are not yet available. We studied the size of the nbM neurons using two sorts of preparations including cresyl violet and the indirect im-

munoperoxidase method with a monoclonal antiacetylcholinesterase (AChE) antibody counterstained with cresyl violet, focusing on both cholinergic and noncholinergic neurons.

MATERIALS AND METHODS

Three patients were histologically verified AD and three age-matched controls were enrolled in this study. The details of the clinical and neuropathological findings of AD patients and the controls are listed in Table 1 and 2 respectively. Case 3 had dominantly inherited AD with juvenile onset and had already been reported by one of the authors (Fukutani et al., 1989). The left cerebral hemisphere containing the Ch4 fixed with 10% formalin for 2 wk was embedded in celloidin convenient for serial preparations. The tissue blocks were coronally and serially cut in 20- μ m slices. The sections for cytometric measurement were carefully chosen at the 100th section for the cresyl violet stain, and at the 101st section for the immunohistochemical stain from the caudal end of the nucleus in both the AD and control cases. The sections examined contained the intermediodorsal and intermedioventral division of Ch4. The immunohistochemical procedure employed the avidin-biotinylated complex method (Vector Laboratory) using a murine monoclonal antibody against AChE (mouse IgG₁, dilution 1:20), which is commercially available from Chemicon Inc. 0.1% diaminobenzidin tetrahydrochloride solution diluted with 0.05M tris buffer supplemented with an equal vol of 0.03% H₂O₂ solution was used as a chromogen. The sections with immunohistochemical stain were also counterstained with cresyl violet. In one preparation, the cross-sectional area of all the Ch4 neurons with clearly visible nucleoli under 400-fold magnification, which were all photocopied using a Nikon Biophoto with Fx-35A, was measured with a computer imaging system (Kontron). The evaluation of data provided a mean value of the cross-sectional neuronal area (CNA; μ m²), number of neurons (NN), standard deviation of CNA (SD; μ m²), and coefficient of variation (CV). Statistical comparisons of these data were made using one-way analysis of variance (ANOVA) followed by multiple comparisons, Kruskal-Wallis's nonparametric ANOVA including Newman-Keul's test for unequal sample sizes.

RESULTS

The cholinergic neurons in the Ch4 of the nbM were identified as immunoreactive products to antibody to AChE in the neuronal perikaryon and partly in the nerve fibers, and were observed in both the control and AD brains (Fig. 1).

Table 1
Clinical and Neuropathological Findings of AD Cases

	Case 1	Case 2	Case 3
Sex	f	m	f
Age at onset, y	55	44	33
Age at death, y	58	50	45
Duration, y	3	6	12
Family history	(-)	(-)	(+)
Clinical findings	General slowing→ visuospatial agnosia, apraxia→ logoclonia→ dementia	Forgetfulness→ gait disturbance→ dementia, inertia, apraxia, upward gaze palsy→ pyramidal tract signs	Paranoid delusion→ dementia→ rigidity, myoclonic jerk→ convulsive seizure→ akinetic mutism
Brain weight, g	990	980	740
Neuropathology	CC: moderate loss of neuron, NFTs, SPs CW: well-preserved myelin with slight gliosis SCG: loss of neuron and gliosis in the amygdala and hippocampus with NFTs CB: slight gliosis in the hemisphere	CC: moderate loss of neuron, NFTs, SPs CW: gliosis in subcortical white matter marked in F1 and parietal lobe SCG: loss of neuron and gliosis in the hippocampus and amygdala with NFTs CB: slight gliosis in the hemisphere	CC: severe loss of neuron, numerous NFTs and SPs rarefaction and spongy state, hypertrophic astrocytes CW: severe loss of myelin and gliosis SCG: severe loss of neuron in the thalamus, striatum, amygdala, and hippocampus with many NFTs and SPs CB: many SPs and loss of Purkinje cell

CC: Cerebral cortex; CW: Cerebral white matter; SCG: Subcortical grey matter; CB: Cerebellum; F1: superior frontal gyrus; NFTs: neurofibrillary tangles; SPs: senile plaques

Table 2
Age, Sex, Cause of Death, and Brain Weight of Three
Normal Controls

	Case 1	Case 2	Case 3
Sex	m	f	m
Age, y	58	67	56
Cause of death	Leukemia	Hepatoma	Lung cancer
Brain weight, g	1400	1250	1403

Fig. 1. (A) AChE-rich neurons in the Ch4 of AD Case 1 and (B) control. Anti-AChE monoclonal antibody, avidin-biotin complex method counterstained with cresyl violet. X400.

Cresyl Violet Preparations

The morphometric data of the cresyl violet preparations in the AD patients and controls are shown in Table 3. Figure 2 shows the neuronal populations by CNA in the three controls and AD patients. The disease duration was 3 y in Case 1, 6 y in Case 2, and 12 y in Case 3. These differences in disease duration between the three AD patients make it possible to determine the time-related changes in the neuronal cell size of the nbM. In Case 2, the Ch4 neurons were not sufficiently stained with cresyl violet. The number of neurons less than $200\ \mu\text{m}^2$ did not significantly change in Case 1, whereas there was a trend toward an increase in the number of neurons less than $200\ \mu\text{m}^2$ in Case 3 owing to atrophy of the neurons more than $200\ \mu\text{m}^2$. But the number of neurons less than $201\ \mu\text{m}^2$ was decreased in Case 2, probably owing to a reduction in cresyl violet staining intensity. The loss of neurons amounted to about 53.3%, and the value of CNA was $240 \pm 156\ \mu\text{m}^2$ versus the CNA value of $299 \pm 160\ \mu\text{m}^2$ of the controls. These findings indicate that the neuronal cell size of $200\ \mu\text{m}^2$ represents the boundary value between affected and nonaffected neurons.

AChE Immunohistochemical Preparations

In the three controls, the values of NN, CNA, SD, and CV of both cholinergic and noncholinergic neurons were similar, and no statistically significant differences between the three controls were found. NN, CNA, SD, and CV of both cholinergic and noncholinergic neurons in the three AD patients and controls are listed in Tables 4 and 5. The frequency distribution of both cholinergic and noncholinergic neurons in each control case and AD case is shown in Figs. 3 and 4. The number of cholinergic neurons in the AD patients decreased in parallel with increasing disease duration. However the CNA of Case 1 was definitely larger than that of the controls ($p < 0.01$) showing that the number of 700–1000 μm^2 -sized neurons was increased. The CNA of the cholinergic neurons

Table 3
Demographic Morphometric Data Obtained From the Cresyl Violet Preparations

Groups	CNA (μm^2)	SD	Number of neurons	CV
Controls	298.6	160.3	2803 ^a	53.7
Case 1	290.0	186.0	572	64.1
Case 2	258.2	143.0	384	55.4
Case 3	170.1	89.1	507	52.4

CNA: cross-sectional neuronal area; SD: standard deviation of CNA; CV: coefficient of variation.

^atotal number of neurons of the three control subjects.

Fig. 2. Neuronal cell population by cross-sectional neuronal area in cresyl violet preparations. Horizontal bar indicates the cross-sectional neuronal area (μm^2) and vertical bar indicates number of neuron. Decrease in the number of 200–700 μm^2 -sized neurons and increase in the number of neurons less than 200 μm^2 become definite in parallel with increasing disease duration. In AD Case 1, the number of neurons more than 700 μm^2 is slightly increased.

Table 4
Results of One-Way ANOVA Followed by Kruskal-Wallis's
Nonparametric ANOVA in the Cholinergic Neuronal Cell Population

Groups	CNA (μm^2)	SD	Number of neurons	CV
Controls	395.2	173.3	1546 ^a	43.9
Case 1	506.7	238.3	204	47.0
Case 2	339.1	154.7	127	45.6
Case 3	219.9	104.0	116	47.3
Newman-Keuls test for unequal neuronal cell sizes sampled ($p < 0.02$)				
Groups	Differ. of means ^b		N-K's q(r,NO) ^c	
Controls & Case 1	111.48	> ^d	54.4737	
Controls & Case 2	56.144		69.0399	
Controls & Case 3	175.308	>	72.2393	
Case 1 & Case 2	167.624	>	69.0399	
Case 2 & Case 3	286.788	>	72.2393	
Case 3 & Case 1	119.164	>	72.2393	

^atotal number of neurons of the three control subjects

^bdifferential of means of CNA

^cNewman-Keuls's q

^d> indicates statistically significant difference ($p < 0.01$).

Table 5
Results of One-Way ANOVA Followed by Kruskal-Wallis's
Nonparametric ANOVA in the Noncholinergic Neuronal Cell
Population

Groups	CNA (μm^2)	SD	Number of neurons	CV
Controls	322.0	181.8	1001 ^a	56.5
Case 1	303.7	186.2	433	61.3
Case 2	200.1	119.2	391	59.6
Case 3	169.2	65.9	426	38.9
Newman-Keuls test for unequal neuronal cell sizes sampled ($p < 0.01$)				
Groups	Differ. of means ^b		N-K's q(r,NO) ^c	
Controls & Case 1	18.388		33.2046	
Controls & Case 2	121.951	>	34.9424	
Controls & Case 3	152.842	>	33.4763	
Case 1 & Case 2	103.563	>	34.9424	
Case 2 & Case 3	134.454	>	33.4763	
Case 3 & Case 1	30.891		34.9424	

^atotal number of neurons of the three control subjects

^bdifferential of means of CNA

^cNewman-Keuls's q

Fig. 3. Cholinergic neuronal cell population by cross-sectional neuronal area in AChE immunohistochemical preparations. The number of 200–700 μm^2 -sized neurons is decreased and the number of neurons less than 200 μm^2 is decreased. Increase in the number of neurons more than 700 μm^2 is noted.

Fig. 4. Noncholinergic neuronal cell population by cross-sectional neuronal area in AChE immunohistochemical preparations. Increase in the number of neurons less than 200 μm^2 and decrease in the number of neurons more than 200 μm^2 are seen in parallel with increasing disease duration. Neuronal cell depletion is not present.

of Case 2 was similar to that of controls, whereas the CNA of Case 3 was smaller than that of the controls ($p < 0.01$).

In contrast, the NN of the noncholinergic neurons did not decrease whereas CNA decreased in parallel with increasing disease duration. There were statistically significant differences in the CNA of the noncholinergic neurons between the controls and Case 2 and between the controls and Case 3 ($p < 0.01$). The value of CV usually expresses the degree of uniformity of the neuronal cell population, and the CV of both cholinergic and noncholinergic neurons were similar, indicating that both small and large neurons in nbM were affected equally.

In summary, the cholinergic neurons in the Ch4 decreased in parallel with increasing disease duration, with some becoming hypertrophic in the early stage, and becoming gradually atrophic with disease progression.

DISCUSSION

The present study provides the evidence that only cholinergic neurons decrease in number, with some of the surviving cholinergic neurons showing hypertrophy in the early stage of AD. In addition, the noncholinergic neurons show only atrophy without neuronal cell depletion. Cytometric research using cresyl violet stain on a normal neuronal cell population of the magnocellular basal forebrain system provided the boundary value of $200 \mu\text{m}^2$ between the small and large neurons in Ch4, with the number of Ch4 neurons more than $200 \mu\text{m}^2$ clearly decreased in the same three AD cases in this study (Kobayashi et al., 1990). The findings obtained from cytometry in the cresyl violet preparations and AChE immunohistochemistry preparations of the Ch4 neurons indicate that the increased number of neurons less than $200 \mu\text{m}^2$ was owing to atrophy of the noncholinergic neurons and that loss of large neurons more than $200 \mu\text{m}^2$ was owing to the atrophy and depletion of the cholinergic neurons. This cytometric finding is consistent with the findings of morphometric analysis of nbM neurons using neuron-specific enolase immunohistochemistry (Allen et al., 1988), which demonstrated an increase in the number of small neuronal cells and decrease in the number of large neuronal cells $200\text{--}799 \mu\text{m}^2$ in size.

The cholinergic neurons in Case 1 were enlarged in size, and the frequency distribution showed a decrease in $50\text{--}699 \mu\text{m}^2$ -sized neurons and increase in $700\text{--}1000 \mu\text{m}^2$ -sized neurons. Some experimental studies have reported that focal neocortical and nbM lesions produce contralateral nbM neuron hypertrophy (Kiyosawa et al., 1989; Pearson et al., 1987). However many morphometric studies have not demonstrated nbM neuron hypertrophy in AD because these studies did not employ the method used in the present study. The finding of nbM neuron hypertrophy in the early stage of AD raises two possibilities regarding the pathological and biochemical processes in AD. One is that nbM

neruon hypertrophy is the result of secondary degeneration from extensive cortical lesions, i.e., axonal reaction of the neuronal cell, and the other is that a compensatory mechanism characteristic of nbM neruons against the disease process of AD promotes enlargement of the cholinergic neuronal cell. Tissue extracts from AD brain contain certain neurotrophic factors that elevate choline acetyltransferase (ChAT) activities (Atterwill et al., 1981) and stimulate growth of central cholinergic neurons (Appel, 1981; Hefti, 1983). Selective neurotrophic effects of NGF on the cholinergic neurons have been reported in several papers. NGF receptor proteins were demonstrated in the central cholinergic neurons (Gnahn et al., 1983; Hefti et al., 1986; Martinez et al., 1987). In addition, AChE immunohistochemistry demonstrated elevated activity of AChE around senile plaques and neurofibrillary tangles (Perry and Perry, 1983), and many neurons of the nbM containing neurofibrillary tangles were documented in a few papers (Hirano and Zimmerman, 1962; Nakano and Hirano, 1985). These findings indicate that in AD the nbM cholinergic neurons are primarily involved, and that their hypertrophic expression is closely associated with preserved sensitivities to specific neurotrophic factors such as NGF, increased levels of NGF receptors, or elevated levels of neurotrophic factors. To resolve this issue requires NGF and NGF receptor immunohistochemistry on the CH4 neurons.

The noncholinergic neurons showed only atrophy, which was more marked in the advanced stage. These morphological alterations showing neuronal cell atrophy without depletion should be interpreted as secondary degeneration probably owing to extensive lesions of the cerebral cortex. These atrophied neurons include other monoaminergic neurons. These atrophic changes of the noncholinergic neurons cause a widespread derangement of neural transmission and may also be responsible for the clinical features of AD.

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