DECREASED NEURONAL ACTIVITY IN THE NUCLEUS BASALIS OF MEYNERT IN ALZHEIMER'S DISEASE AS SUGGESTED BY THE SIZE OF THE GOLGI APPARATUS

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Abstract—In order to study changes in neuronal activity in the nucleus basalis of Meynert in aging and Alzheimer's disease, we applied a polyclonal antibody directed against the Golgi apparatus on formalin-fixed, paraffin-embedded material. Subsequently, an image analysis system was used to measure the size of the Golgi apparatus in (i) all nucleus basalis neurons and also separately in (ii) the remaining large cells (perikaryonal diameter > 30 μ m). A significant reduction of 49% in the size of the Golgi apparatus was found in the entire population of nucleus basalis neurons in Alzheimer's disease. Furthermore, although there was no significant decrease in the size of the persisting large neurons in the nucleus basalis of Meynert, a significantly decreased size of the Golgi apparatus was found in these neurons in Alzheimer's disease.

These results suggest that the overall activity of nucleus basalis neurons is severely decreased in Alzheimer's disease. Furthermore, these data support the idea that atrophy and decreased activity are the main phenomena in the nucleus basalis in Alzheimer's disease; they also indicate that the size of the Golgi apparatus is a sensitive parameter to follow this process.

The nucleus basalis of Meynert (NBM), or the Ch4 division according to Mesulam's nomenclature,³⁴ is one of the most prominent subcortical structures in the human basal forebrain. Together with the septal nuclei and the diagonal band of Broca, it is the major source of cholinergic innervation to the hippocampus, amygdala and cerebral cortex.^{20,34,37,42,55}

The NBM is severely affected in Alzheimer's disease (AD),^{6,9,13,31,35,36,55,57} as well as in other neurological disorders with deterioration of memory and cognitive functions, e.g. Creutzfeldt–Jakob's disease,⁷ Parkinson's disease,^{6,59} Pick's disease,⁵² Korsakoff's disease^{6,39} and progressive supranuclear palsy.⁵¹ When using markers (e.g. Alz-50) for cytoskeletal alterations, the NBM of AD patients displays a very clear staining of the perikarya and dystrophic neurites, in contrast to controls.^{48,53}

Estimations of the neuronal numbers of the NBM during normal aging vary greatly, i.e. from a loss of 23–70%^{29,32} to no neuronal loss at all.^{8,56} Originally, it was presumed that massive cell death is one of the major hallmarks of AD in the NBM.^{6,55,57} However, recent studies have indicated that neuronal atrophy rather than cell loss is the main phenomenon in the NBM in AD.^{2,38,43} The concept of neuronal atrophy rather than cell death being the main hallmark of AD

may have important consequences for future therapeutic strategies.^{41,49}

In order to establish whether changes in neuronal activity are present in the NBM in AD, we estimated the activity of the NBM neurons using the size of the Golgi apparatus (GA) as a sensitive parameter for neuronal activity. The GA has a central function in intracellular transport, processing and modification of secretory products, plasma membrane and lysosomal proteins.^{14,19} Furthermore, it had been shown before that the size of the GA shows a clear relation with changes in cell activity.^{27,30,46,47,54} In the present study the GA was visualized immunocytochemically, after which the size of the organelle was measured using an image analysis system in order to establish (i) whether decreased neuronal activity is present in the NBM in AD and (ii) whether there is a compensational activity due to atrophy of nearby neurons.

EXPERIMENTAL PROCEDURES

Tissue collection

Brains from 14 control subjects ranging in age from 32 to 95 years without a primary neurological or psychiatric disease and from 10 Alzheimer patients ranging in age from 40 to 90 years were obtained at autopsy (see Table 1 for details). Neuropathological investigation of the controls and confirmation of the diagnosis of AD was performed by Dr W. Kamphorst (Free University) and Dr D. Troost (Academic Medical Centre), both in Amsterdam. After weighing the brain, the hypothalamus containing the NBM was dissected out and fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.4) at room temperature,

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Abbreviations: AD, Alzheimer's disease; GA, Golgi apparatus; NBM, nucleus basalis of Meynert; NGFr, nerve growth factor receptor; TBS, Tris-buffered saline.

Table 1. Clinical and neuropathological information on controls and Alzheimer patients

Autopsy no.	Sex	Age	Fixation time (days)	Pmd (h)	Brain weight (g)	Clinical diagnosis/cause of death
<i>Controls</i>						
(1) 864261	m	32	11	49	1440	AIDS, pneumonia
(2) 84018	f	36	51	96	1420	Multiple fractures
(3) 81012	f	38	47	3	1360	Cervix carcinoma
(4) 83173	f	46	33	<11	1360	Adrenal carcinoma
(5) 80048	f	46	n.r.	2.5	1300	Ovary carcinoma
(6) 88311	m	49	49	<24	1309	Hepatic failure
(7) 82165	f	52	33	5	1370	Mamma carcinoma
(8) 9246	f	54	n.r.	13	1080	Traffic accident
(9) 80087	f	60	198	24	1110	Acute leukaemia
(10) 81014	f	64	44	8	1090	Renal insufficiency
(11) 80118	m	70	n.r.	<24	1165	Pneumonia, cerebrovascular accident
(12) 82175	m	85	44	16	1400	Chronic myelocytic leukaemia, pneumonia
(13) 9312	m	92	32	11	1038	Myocardial infarction
(14) 8550	f	95	n.r.	n.r.	n.r.	n.r.
Mean $\pm$ S.E.M.	58.5 $\pm$ 5		54.2 $\pm$ 16	21.0 $\pm$ 7	1264.7 $\pm$ 40	
<i>Alzheimer cases</i>						
(15) 89166	m	40	28	3	1370	AD, cachexia
(16) 86364	m	45	119	4	1330	Familial AD, cachexia
(17) 89345	m	52	102	23	1296	AD, decompensatio cordis
(18) 86196	m	61	63	84	1260	AD
(19) 88325	f	64	35	3	800	AD, cachexia, dehydration
(20) 8560	f	70	34	30	1200	AD, lung embolism
(21) 88302	f	78	31	n.r.	1240	AD, arthrosis
(22) 8563	f	81	35	40.5	1165	AD, bronchopneumonia
(23) 8484	m	87	292	42	1275	AD, pneumonia, diabetes, hypothyroidism
(24) 84050	f	90	33	2	860	AD, anaemia
Mean $\pm$ S.E.M.	66.8 $\pm$ 5		77.2 $\pm$ 26	25.7 $\pm$ 2	1179.6 $\pm$ 61	

n.r., not recorded; Pmd, *post mortem* delay.

generally for one month. Subsequently, tissue was routinely dehydrated and embedded in paraffin. Serial 6- μ m coronal sections were cut and mounted on chrome-aluminium sulphate-coated glass slides. For anatomical orientation, every fiftieth section was stained with 0.1% Thionine in acetate buffer (pH 4.0).

Immunocytochemistry

As primary antibody a polyclonal organelle-specific antibody was used which was raised against immunoaffinity purified MG-160, a sialoglycoprotein of the medial cisternae of rat neuronal GA (160,000 mol. wt).^{10,17} The specificity of this antibody has been established previously by light and immunoelectron microscopy.^{10,17,18} Applied to microwave-pretreated sections, it has proved to be a reliable reagent for the immunocytochemical visualization of the GA in human brain fixed in formalin and embedded in paraffin for prolonged periods of time.^{10,18,30} For optimal retrieval of the MG-160 antigen, we used microwave oven heating before incubation with the primary antibody, after Shi *et al.*⁴⁴ and as described in Lucassen *et al.*³⁰ Since vigorous boiling during microwave treatment caused detachment of the sections, we used a thermo probe (Miele) to prevent the temperature from rising above 95°C.

Briefly, mounted sections were hydrated and stained using the following procedure: (i) incubation of the sections in a microwave oven (Miele electronic M-696) in a solution of 1% ZnSO₄ for 2 $\times$ 5 min at a maximum of 95°C; (ii) washing in Tris-buffered saline (TBS; pH 7.4) for 2 $\times$ 10 min; (iii) pretreatment of the sections in a 0.5% saponin (Merck) and 3% fish gelatin (Merck) solution in water for 30 min; (iv) preincubation in 10% horse serum in TBS (10 min); (v) incubation with the primary antibody (MG-160), 1:800 in phosphate-buffered saline (pH 7.6), for 1 h at room temperature followed by overnight incubation at 4°C; (vi) washing in TBS (2 $\times$ 10 min); (vii) incubation with biotinylated goat anti-rabbit immunoglobulin (Vector) 1:500 for 1 h at room temperature; (viii) washing in TBS (2 $\times$ 10 min); (ix) incubation with avidin-biotin-horseradish peroxidase

(Vector Elite kit) 1:1500 in supermix (0.5 M Tris, 1.5 M NaCl, 2.5% gelatin and 5% Triton X-100 (Sigma) for 1 h at room temperature; (x) washing in TBS (2 $\times$ 10 min); (xi) rinsing in Tris-HCl (pH 7.6); (xii) incubation in 0.05 mg/ml 3,3'-diaminobenzidine (Sigma) in a total volume of 15.5 ml 0.05 M Tris-HCl (pH 7.6) containing 5 μ l 30% H₂O₂ and 0.035 g ammonium nickel sulphate¹ at room temperature for 10 min; (xiii) rinsing in TBS, followed by (xiv) dehydration in graded ethanol and xylene. The sections were subsequently coverslipped with Entellan (Merck).

Morphometry

Since the NBM is a very extensive cell system, the measurements were performed in a standardized part of the NBM, i.e. in the medial and lateral subdivisions of Ch4a, according to Mesulam's nomenclature.³⁴ NBM-containing sections were selected on the basis of a standardized location of the fornix, anterior commissure, optic tract and supraoptic nucleus.¹³

Measurement of the GA surface was performed using an IBAS-KAT image analysis system (Kontron KAT-based system). The image analysis system was connected to a Bosch TYK9B TV camera equipped with a chalnicon tube mounted on a Zeiss microscope. The microscope was equipped with planapo objectives. All measurements were performed using a 560-nm small band DEPAL filter (Schott, Germany) which coincides with the maximum absorption of the diaminobenzidine/nickel sulphate precipitate in the sections. For each section, the analysis consisted of one of the two following procedures. (1) Random sampling of NBM neurons regardless of their size and measurement of the GA area in those neurons in which the nucleolus was present. (2) Selective sampling of large neurons (perikaryonal diameter > 30 μ m) based on the Whitehouse criteria³⁷ (which is equivalent to a cross-sectional area of 370 μ m²) and subsequent measurement of the cell and the GA areas of these cells.

Random sampling consisted of the following two steps: (i) area selection and (ii) determination of the total GA

Fig. 1(A-D)—caption overleaf.

Fig. 1(E–G).

Fig. 1. Illustration of random sampling of NBM profiles using $\times 2.5$ (A–D) and $\times 40$ (E–G) objectives. Selection of the NBM area (A), manual outlining of the NBM (B), superimposing a grid on the selected area (C), random sampling of the fields (D), finding the profiles in the selected fields using the $\times 40$ magnification (E), formation of a mask for the stained GA (note the similarity with the GA in the original section in E), (F) and outlining of the selected profiles (G), after which calculation of the Golgi area was performed. n, nucleolus. Scale bars = 0.5 mm (A–D); 30 μm (E–G).

Fig. 2. Immunocytochemical staining of the GA in young (A; no. 84018 in Table 1) and old (B; no. 82175) controls and AD patients (C, D; nos 84050 and 8484, respectively). Note the clear reduction in size of the GA in AD patients when compared to the old controls. Scale bar = 30 μ m.

area per cell profile of all the neuronal profiles containing a nucleolus. Sampling and area selection was performed as follows: in each section to be analysed, an area widely covering the NBM (using the $\times 2.5$ objective of the microscope) was loaded into the IBAS and displayed on the image analysis monitor (Fig. 1A). The position of the section under the microscope was stored using the X - Y coordinates of the scanning stage. In this image, the contour of the NBM was outlined manually (Fig. 1B). In order to select a number of fields in the NBM, a grid which consisted of areas corresponding to the image size at a $\times 500$ magnification ($\times 40$ objective) was superimposed over the NBM area (Fig. 1C).

From this grid, 15 fields were selected randomly (Fig. 1D). The position of each microscopic field belonging to this sample was again expressed in the X - Y coordinates of the scanning stage. On the basis of these coordinates, the selected fields were retrieved for measurement.

In order to determine the total GA area per cell profile, the $\times 40$ objective was positioned in the microscope and the scanning stage moved to the previously defined positions of the high magnification measuring areas. At each position, a 768×512 pixel large image was loaded and stored on an optical disk (Corel drive Panasonic) (Fig. 1E). Subsequently, a mask of the GA area in the selected cells in all stored images was calculated (Fig. 1F). Each of the gray value images with the calculated GA masks was then retrieved under programme control and all profiles containing a nucleolus were outlined manually (Fig. 1G). These outlines were stored together with the Golgi masks. Finally, the total area of the GA was calculated. For the selective sampling of the large neurons, cells were outlined manually and GA as well as cell profile areas were calculated. The data were stored per section per patient per group in an ascii file and in a Quattro Pro compatible file on diskette.

Statistical methods

The differences in mean values of the neuronal GA and cross-sectional areas between controls (young and old) and AD groups were tested using the Mann-Whitney non-parametric test. In addition, the data on the size of the GA of all NBM neurons of all AD patients ($n = 10$) were pooled and compared with their age-matched controls ($n = 12$). Furthermore, frequency distributions of these values were mutually compared using the chi-square test. In order to test the correlation between the different parameters (fixation time, *post mortem* delay, age of the subjects and GA area) the P -Pearson test was used. A P -value < 0.05 was considered to be significant.

RESULTS

Using the microwave pretreatment, we were able to retrieve the MG-160 epitopes of the GA from NBM neurons in sections of formaldehyde-fixed and paraffin-embedded material. In both controls and AD patients, immunocytochemical visualization of the GA in the NBM neurons revealed a prominent and intense cytoplasmic staining with a perinuclear distribution in both neurons and glia, consistent with the location of the GA (Fig. 2A-D). Qualitative microscopic analysis had already shown that the area occupied by the GA in the cytoplasm of NBM neurons is generally smaller in AD patients (Fig. 2C, D) than in controls (Fig. 2A, B).

Measurements confirmed that the majority of controls have larger GA areas than AD patients (Fig. 3). However, the differences in size of the GA between controls and AD patients became smaller in the oldest age groups, since in controls the size of the GA

decreases with age ($Y = -1.05X + 172.7$, $r = -0.57$, $P < 0.05$). No such correlation was observed in the AD group (Fig. 3). Furthermore, no explanation can be given for the few AD patients with a relatively large GA (nos 18, 21 and 22; see Table 1).

No significant correlation could be found between fixation time and *post mortem* delay and the mean values of the GA area in both control and AD groups ($P < 0.2$). In addition, the data on the size of the GA of all NBM neurons of all AD patients were compared with those of their age-matched controls. When dividing the AD group into a senile and presenile group on the basis of their age (65 years), no significant differences were observed between these two groups ($P < 0.1$).

As mentioned in the morphometry section, measurements were performed in two populations of NBM neurons.

All neurons containing a nucleolus regardless of their size

Control versus Alzheimer's disease subjects. The frequency distribution of the GA area showed a highly significant reduction in size of the GA in AD patients compared to controls ($P < 0.001$) (Fig. 4A). The mean GA area per neuron per patients was $110.2 \pm 10.6 \mu\text{m}^2$ (mean $\pm$ S.E.M.) in the control group and $56.6 \pm 11.4 \mu\text{m}^2$ in the AD group, i.e. a 49% reduction in this condition ($P < 0.001$).

Young versus old controls. No significant difference in the mean value of the GA area per cell profile was found between young and old controls ($P < 0.2$). The frequency distribution of the GA area in old controls shifted only slightly, but not significantly to the left ($P < 0.1$) (Fig. 4B).

When dividing the AD group into a senile and presenile group on the basis of their age (65 years), no significant differences were observed between these two groups ($P < 0.1$).

Second group

This group consisted of large neurons with a cross-sectional area $> 370 \mu\text{m}^2$. In this group both

Fig. 3. The mean GA area per neuron in various age groups of controls and AD patients. Note the clear difference in size of the GA between controls and AD patients.

Fig. 4. (A) Frequency distribution of the size of the GA in all NBM neurons in controls and AD subjects. The distribution of the GA area has shifted significantly ($P < 0.001$) to lower digits in AD, indicating a strong decrease in the size of the GA. (B) A similar histogram for young and old control subjects does not show any significant shift in the size of the GA to lower digits in old subjects ($P < 0.1$).

the cross-sectional area of the cell and the size of the GA area were measured.

Control versus Alzheimer's disease subjects. The mean size of the cell area in the large cells of the control group was similar to that of the AD group, i.e. 547.48 ± 21 and $507.74 \pm 23.2 \mu\text{m}^2$, respectively. The frequency distribution of the cell area in controls did not show any significant differences compared to the AD group ($P < 0.2$) (Fig. 5A). In contrast, the frequency distribution of the GA area in this group of cells had shifted significantly to the left ($P < 0.001$) (Fig. 6A).

Young versus old controls. The frequency distribution of the cross-sectional area in old controls had shifted slightly but significantly ($P < 0.05$) to the left compared to that of young controls (Fig. 5B). However, no significant shift ($P < 0.2$) was observed in the frequency distribution of the GA area in old versus young controls (Fig. 6B).

DISCUSSION

The first reports claiming a severe neuronal loss in the NBM in AD^{6,55,57} were followed by a number of other publications in which the amount of cell loss in the NBM was reported to vary from a 75% cell loss¹³ to no neuronal loss at all.³⁸ It has been presumed that this controversy is at least partly due to the heterogeneity of the different subdivisions of the NBM.²⁶ However, even studies performed on one particular subdivision of the NBM varied considerably. For instance, measurements performed in the Ch4a area showed differences varying from a 54% cell loss³¹ to no cell loss at all.³⁸

The most likely explanation for these equivocal results is the use of different criteria for the size of counted cells, which is of particular interest considering the atrophy NBM neurons appear to undergo in AD. Mann *et al.*,³¹ for instance, only counted cells with a diameter larger than 30 μm and reported a 54% cell loss in the NBM, whereas Pearson *et al.*³⁸

Fig. 5. (A) The frequency distribution of the size of the remaining large neurons (perikaryonal area $> 370 \mu\text{m}^2$) does not differ significantly between control and AD subjects ($P > 0.2$). (B) A similar histogram for young and old control subjects shows a moderately but significantly decreased cell size ($P < 0.05$) in aging ($P < 0.05$).

Fig. 6. (A) Frequency distribution of the size of the GA in the remaining large neurons (perikaryonal area > 370 μm^2) in control and AD subjects, showing a significant decrease ($P < 0.001$) in the size of the Golgi area in AD. Note the small proportion of large neurons in AD patients that have a large GA (> 210 μm^2), which may be considered to show compensatory activation. (B) A similar histogram for young and old control subjects did not show any significant differences ($P < 0.2$) between these two groups.

counted all NBM neurons regardless of their size and did not find any significant cell loss in the NBM. Whereas the number of large neurons decreases, the number of small neurons increases in AD.^{2,43,58} For this reason, the general concept of major cell loss in the NBM of AD patients had to be abandoned and was replaced by the opinion that neuronal atrophy rather than cell death is the major hallmark of AD in the NBM.^{38,43,49}

Since the size of the GA has been shown before to be a sensitive parameter for neuronal activity both in animal experiments^{27,46,47} and in the human hypothalamus,³⁰ the strong decrease in GA size (49%) observed in the present paper strongly suggests that the capacity of NBM neurons to process and target proteins is severely decreased in AD. This conclusion is consistent with studies showing a decreased volume of the nucleolus as an index for

the protein synthetic capacity of NBM neurons in AD.^{31,50} In addition, it agrees with earlier studies providing evidence for a decrease in the activity of the enzymes choline acetyltransferase and cholinesterase in the NBM in AD,^{4,13,32,39,40} suggesting an overall decline in the protein metabolism of NBM neurons in AD.

On the basis of observations in other brain structures, it has been suggested that in aging and AD, neuronal loss may trigger remaining cells to increase their perikaryal size and induce the proliferation of dendritic branching so that the same volume of the total dendritic tree is maintained.^{5,9,15,25} If this is a general compensatory mechanism, it may be expected that the remaining large NBM neurons in AD become larger in order to compensate for the loss of activity of nearby neurons. However, other studies have already shown that in the population of large NBM neurons, the mean value of the cross-sectional area of the large cells does not change significantly in AD.^{13,28} Our results show that the mean value of the cross-sectional area of the population of large cells does not alter significantly (8%), which is in agreement with the literature values in AD patients, which vary between 2%²⁸ and 14%.¹³ However, unlike the similar mean values of the GA area in this group of neurons, the frequency distribution of the GA area shifts significantly to the left (Fig. 6A), indicating that the majority of the large NBM neurons are metabolically impaired rather than showing a compensatory activation for atrophy of nearby neurons. Yet, a small number of neurons with an extremely large, presumably extra active Golgi area (> 210 μm^2) is present in AD. (Fig. 6A). It is, however, questionable whether so few compensatory active large neurons have a clear functional impact. The decrease in size of the large NBM neurons in normal aging (Fig. 3) has been reported before.¹² However, since no significant difference was found in the frequency distribution of the GA area between young and old controls, the slight shrinkage of these neurons in normal aging does not seem to be accompanied by dramatically decreased neuronal activity.

It has been suggested that cell loss or neuronal atrophy in AD is due to lack of effect of certain trophic factors.^{22,23} However, the major trophic factor for the NBM, i.e. nerve growth factor, as well as its mRNA, are present in normal^{3,16,21} or even higher quantities¹¹ in the cerebral cortex of AD patients than in that of controls. It therefore does not seem to play a role in the pathogenesis of AD.²⁴ On the other hand, it has been shown, at least in rat, that the cholinergic neurons in the basal forebrain contain both low (p75) and high affinity (trkA) nerve growth factor receptors (NGFRs).^{33,45} Because processing of newly synthesized NGFr takes place in the GA, the decreased size of the GA in AD, as reported in the present paper, can be associated with decreased production of the NGFr. This is in agreement with the extensive loss of NGFr gene expression in the NBM neurons.²⁴

The question whether (i) a decreased ability of the NBM neurons to synthesize and process the NGF in AD is a primary event followed by atrophy of the neurons or (ii) alternatively, neuronal atrophy due to a different cause is the primary event followed by a decreased size of the GA and diminished production of NGF cannot be answered at present.

CONCLUSIONS

Our data are consistent with the hypothesis that, in AD, judging from the significant decrease in the size of the GA, synthetic and processing activities of the NBM neurons are severely affected in AD. Even the remaining large neurons generally show a decrease in the size of the GA, consistent with a lower rate of protein processing in AD rather than compensatory

activation. Thus, decreased neuronal activity seems to be the major hallmark of AD in the NBM. The size of the GA can be used as a sensitive parameter to monitor this process.

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