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Resistance to Alzheimer's pathology is associated with nuclear hypertrophy in neurons

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Abstract

This study focuses on the morphometric changes of neurons in asymptomatic Alzheimer's Disease (AD), a state characterized by the presence of AD lesions in subjects without cognitive impairment. In autopsy brains, we used stereological methods to compare the cell body and nuclear volumes of anterior cingulate gyrus (ACG) and CA1 hippocampal neurons in asymptomatic AD subjects (n=9), subjects with AD dementia (AD, n=8), Mild Cognitive Impairment (MCI, n=9), and age-matched controls (Controls, n=9). In ACG, we observed a significant decrease in the neuronal volume of MCI and AD compared to Controls; by contrast, no atrophy was present in asymptomatic AD. Moreover, we found a significant increase in nuclear volume in asymptomatic AD compared to Controls ($P<0.001$), MCI ($P<0.01$) and AD ($P<0.001$) brains. Similar results were found in the CA1 region of the hippocampus. This nuclear hypertrophy may represent an early neuronal reaction to A β or Tau, or a compensatory mechanism which forestalls the progression of AD and allows the brain to resist the development of dementia.

Keywords

normal cognition; stereology; nucleus; hypertrophy

1. Introduction

A longstanding riddle in the investigation of Alzheimer's disease (AD) has been the finding of substantial numbers of A β plaques in the autopsy brains of some individuals with documented normal cognition [54] [48] [34] [23] [12] [43]. This finding has led some researchers to doubt the relevance of A β plaques to the neurodegeneration and clinical

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Disclosure Statement

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manifestations of AD [53]; whereas others have proposed that the brains of certain individuals have a “reserve” that allows them to resist the ravages of the pathological lesions [22] [52] [47]. In the present study, we present evidence that this resistance to the lesions of AD is associated with nuclear hypertrophy of cortical and hippocampal neurons.

The motivation for the present study was to search for morphometric parameters underlying the resilience to AD in cognitively normal subjects with substantial numbers of A β plaques and NFTs in postmortem brain examination, a state previously called pre-clinical AD [54] [48] [34], high pathology controls [26], or high plaque non-demented [2]. Since we do not know *a priori* whether these subjects will remain cognitively intact or eventually progress into MCI or dementia, we believe they are more accurately classified as asymptomatic subjects with AD pathology (asymptomatic AD). Understanding the nature of neuronal changes during this asymptomatic state may shed light on mechanisms that forestall the progression of the disease, allowing some subjects to remain cognitively spared, whereas others with comparable brain pathology progress into mild cognitive impairment (MCI) or dementia. In the present study, we focus on the volumes of the cell body and nucleus of neurons in the anterior cingulate gyrus (ACG) and CA1 region of hippocampus in autopsy brains from subjects with asymptomatic AD in the Baltimore Longitudinal Study of Aging (BLSA) [50]. The decision to examine the ACG was motivated by a longitudinal MRI study of BLSA subjects showing a relatively enhanced vulnerability of this region to cortical atrophy [45]. The hippocampal CA1 region was chosen due to its selective and specific vulnerability to AD [58,59]. We selected for comparison the brains of age-matched subjects with no cognitive impairment (Controls), mild cognitive impairment (MCI), and AD dementia (AD).

2. Materials and methods

2.1 Subjects

Clinical and neuropsychological characterization of subjects—Annual evaluations include a series of neuropsychological tests [24], neurological exam, interval medical history, medication review, and a structured informant and subject interview. For the last 8 years the vehicle for the structured interview was the Clinical Dementia Rating (CDR) [21,33] Diagnosis of Dementia: All subjects were followed annually and were reviewed at a consensus conference if their Blessed Information Memory Concentration score [3] was 3 or above, if their informant or subject CDR score was 0.5 or above or if their Dementia Questionnaire [51] was abnormal. All subjects, regardless of screening tests, were evaluated at a diagnostic conference at time of entry into the autopsy study and at death or withdrawal. The full panel of neuropsychological diagnostic tests and clinical data were available for review at the diagnostic conference. Diagnoses of dementia were based on DSM-III-R criteria [1]. A diagnosis of cognitive impairment not meeting criteria for dementia, analogous to Mild Cognitive Impairment (MCI) [40], was made for participants who had deficits in either a single cognitive domain (usually memory) or had more than one cognitive deficit but did not have functional loss in activities of daily living.

Neuropathological material—We examined 35 post-mortem brains from BLSA participants. The BLSA and autopsy studies are approved by the local institutional review board, and all participants gave written informed consent prior to each assessment. Based on clinical data, including CDR within the last year of life and the neuropathological criteria of the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) [30] the brains were divided into four groups: 1) age-matched controls (Controls): subjects with normal cognition (CDR 0) and no significant AD pathology, 2) asymptomatic AD: subjects with normal cognition (CDR 0) and AD pathology, 3) Mild Cognitive Impairment (MCI): subjects with CDR 0.5 and AD pathology [40], and 4) Alzheimer dementia: subjects with dementia (CDR

≥ 1). All brains were free of significant cerebrovascular lesions, α -synuclein lesions or pathological changes indicating other etiologies of dementia. Table 1 describes the demographic characteristics and neuropathological scores of the sample. The severity of neuritic plaques was assigned a semiquantitative and age-adjusted score (0, A, B, or C) according to the guidelines of CERAD. The staging of NFT was assigned a score (0 to 6) according to the Braak system [6]. As shown in the table, subjects in the asymptomatic AD group had pathological CERAD neuritic plaque scores B (n=5) or C (n=4). Mean brain weights were as follows: Controls = 1302.0 ± 181.1 grams, asymptomatic AD = 1292.0 ± 114.5 grams, MCI = 1264.0 ± 93.1 grams, AD = 1235 ± 96.51 grams. Statistical analysis did not show any significant difference among groups for brain weight. Groups did not differ significantly in mean age, although there was a trend for the AD group to be older.

2.2 Histology

Anterior Cingulate Gyrus. A cylindrical punch of formalin-fixed tissue (4-mm diameter and 10-mm long), including gray and subjacent white matter was taken perpendicular to the brain surface from Brodmann area 24b' [57] (Anterior Cingulate Gyrus) at the level of the genu of the corpus callosum. (Fig. 1A & B) The punch was taken from the exact same area and at the same level on every brain. Vertical histological sections [20] were prepared by cutting the tissue cylinder longitudinally in half, processing, embedding in paraffin, cutting serially at 50 μ m (yielding ~20 sections), and staining with Cresyl Violet (CV). The hippocampus was cut serially on the coronal plane at 50 μ m. We selected two sections, 500 μ m apart, at the level of the Lateral Geniculate Body, and stained them with CV. Cerebellum also was examined in a subset of brains listed in Table 1 (#). The morphometry of Purkinje cells was examined in 10 μ m paraffin sections of the cerebellar hemisphere stained with H&E.

2.3 Immunohistochemistry

ACG tissue sections (10 μ m thick) from all brains were immunostained for A β (6-E-10, Signet Labs, Inc; dilution 1:500) and phosphorylated Tau-protein (PHF-1 clone, a gift from Dr. P. Davies, Albert Einstein College of Medicine, Bronx, NY; dilution 1:100). These 10 μ m sections were used only to assess the fractional areas (loads) of A β and Tau immunoreactivities and not to measure neuronal or nuclear volumes.

2.4 Unbiased Stereology

For unbiased stereological analysis of ACG neurons, we examined a subset including every other vertical section (n=10). Using the Stereo Investigator Optical Dissector software from MBF Biosciences, MicroBrightField, Inc. (Williston, VT, USA), we outlined a contour (Fig. 1C) of cortical gray matter and selected sampling sites with a grid size = $700 \times 700 \mu$ m and a "disector" height of 35 μ m, with a 2- μ m guard zone. Then, using the Vertical Nucleator probe, we measured the volume of neuronal cell bodies and nuclei by placing 4 rays through the nucleolus. This was performed with a 100X, NA 1.30, oil Uplan FL $\infty/0.17$ objective. The image was captured with a video camera Hitachi HV-C20 3CCD. We measured between 164 and 248 neurons per each ACG. Cortical neurons were measured irrespectively of layer and shape. For the hippocampus the grid size was $250 \times 250 \mu$ m and we measured between 201 and 206 CA1 neurons per brain. In addition, we also measured the cell body and nuclear volumes of 100 consecutive Purkinje neurons in the cerebella of 24 subjects from all groups. The fractional areas of A β and Tau-protein immunoreactivity were measured in the ACG using the Area-Fraction-Fractionator probe (grid size: $450 \times 450 \mu$ m), which allowed us to assess the burden of those proteins in the sections. All assessments were performed blinded to clinical and neuropathological diagnoses. The criteria for selecting neurons to be measured included a well defined perikaryon, nucleus, and nucleolus. Neurons were measured if their nuclear membrane intersected or touched the inclusion (green) line and excluded if the nuclear

membrane intersected or touched the exclusion (red) line. The measuring rays were cut at the intersection with the cell or nuclear membrane. (Fig. 1D) If a ray extended into a dendrite, the ray was cut at the base of the dendrite.

2.5 Statistical analysis

We performed analyses of variance (ANOVA) with PROC GLM (SAS v9.1-3, SAS Institute, Cary, North Carolina, USA) with contrasts among the four groups. Separate analyses were conducted for neuronal cell body volumes and nuclear volumes for the ACG and Hippocampus CA1. Differences among groups in the burden of A β and Tau in the ACG were also examined. Group differences were considered significant at $p < 0.05$ with Tukey-Kramer adjustment for multiple comparisons.

3. Results

Lack of neuronal atrophy in asymptomatic AD brains

In this study we focused on the brains of asymptomatic AD subjects, characterized by normal cognition despite AD lesions severe enough to cause MCI or dementia in other individuals. The size of ACG and CA1 neuronal cell bodies in asymptomatic AD were compared to that of Controls, with whom they share normal cognitive status, and to that of MCI and AD (Fig. 2, Fig. 3 and Fig. 4), with whom they share comparable A β and Tau lesions (Fig. 5). We anticipated that, as consequence of the AD pathology, there would be a decrease in the size of neurons in asymptomatic AD compared to Controls. Contrary to this expectation; neuronal volume in neither ACG nor CA1 was significantly different in asymptomatic AD compared to Controls (Table 2, Fig. 2, Fig 3 and Fig. 4). However, the MCI and AD groups showed significant ACG neuronal atrophy compared to asymptomatic AD (20.6%, $P < 0.05$; and 32.2% $P < 0.001$, respectively). Compared to the Controls group, the AD group showed significant neuronal cell body atrophy (23.1%, $P < 0.05$), whereas the smaller volume in MCI was not significant (10.0%, $P > 0.05$). (Table 2 and Fig. 2B) Similar findings were noted in CA1 neurons (Table 2 and Fig. 4B), but were absent in the Purkinje neurons of the cerebellum (Table 2).

Hypertrophy of neuronal nuclei characterizes asymptomatic AD brains

In the brains of asymptomatic AD subjects there was a striking nuclear hypertrophy of ACG and CA1 neurons, which separated asymptomatic AD from both Controls and MCI. ANOVA revealed significant differences overall in mean neuronal nuclear volumes for the ACG (Table 2 and Fig. 2A) among the four groups ($P < 0.001$). In asymptomatic AD we observed a significant increase in the mean ACG nuclear volume compared to the Controls (42.8%, $P < 0.001$), MCI (28.5%, $P < 0.01$), and AD (64.0%, $P < 0.001$) groups (Table 2 & Fig. 2A). A similar nuclear hypertrophy was present in CA1 neurons of asymptomatic AD, with significant differences among groups in mean CA1 nuclear volumes ($P < 0.001$). CA1 nuclear volumes were increased in asymptomatic AD compared to Controls (39.1%, $P < 0.05$) and AD (25.9%, $P < 0.05$) (Table 2 & Fig. 4A). In contrast, there were no significant differences among groups for volumes of the nucleus of Purkinje cells (Table 2).

In terms of the burden of A β and Tau-protein in the ACG, we did not observe significant differences in the fractional areas of immunoreactivity between the asymptomatic AD and MCI groups. However, both parameters were significantly increased in AD subjects compared to each of the other groups (Fig. 5).

4. Discussion

Our results provide a novel finding of nuclear hypertrophy of ACG and CA1 neurons in asymptomatic AD in comparison to age-matched Controls, as well as MCI and AD. Moreover,

the volume of the neuronal cell bodies in asymptomatic AD did not differ significantly from controls without significant AD pathology. By contrast, the volume of the neuronal cell bodies showed significant atrophy not only in AD but also in MCI.

We limited our study to males because most of the BLSA participants who have come to autopsy to date are males, in part due to their earlier enrollment into the BLSA, and will extend these analyses to females in the future. To avoid confounding effects of non-AD pathology, we excluded subjects with cerebrovascular disease, α -synuclein pathology or other dementias. To examine whether the morphometric changes observed in ACG and CA1 neurons were generalized throughout the brain or could be artifactual in nature, we also measured the volumes of cerebellar Purkinje cell bodies and nuclei in a subset of subjects and found no differences among the groups. There were no significant associations among the volumes of neurons and nuclei and postmortem interval or time of the specimen in fixative.

Previous studies of the neuropathology of asymptomatic AD (preclinical AD) have described and quantified A β plaques [32] and neurofibrillary tangles (NFT) [17] [31] [18] [8] and also have shown that the number of neurons remains stable in hippocampus and entorhinal cortex [59] [42]. Our observations indicate that, whereas the size of neuronal cell bodies in asymptomatic AD is comparable to that of age-matched controls, the nuclear volume is markedly and significantly increased compared not only to old controls (42.8% in ACG, 39.1% in CA1 neurons), but also to the MCI and AD groups. (Fig. 2A and Fig. 4A). The increase in nuclear size in subjects who have A β deposits in the brain but remain cognitively intact may suggest a cellular response to injury. Nuclear hypertrophy may reflect the reactive and compensatory processes at the cellular level that allow the brain to function normally despite the presence of AD lesions, or may represent the earliest stages of a neurodegenerative response. Furthermore, it is attractive to speculate that those individuals whose brain cannot mount such a response during initial stages of AD pathology are the ones who will progress to MCI and dementia.

The observed increase in nuclear volume in asymptomatic AD is reminiscent of the pathological response to injury [4] or axotomy [29] and our findings may represent an increased metabolic activity of neurons as suggested by others [14] or being a direct or indirect effect of A β toxicity [13]. Another possible explanation for our observations of increased nuclear volume could be the entrance of neurons into cell-cycle as described in hippocampal neurons in AD and MCI patients [19] [60] [56]. The observed increase in nuclear size could be consistent with the reported increase in nucleo-cytoplasmic of cells in the S and G2 phases [5] [37].

Increases in nuclear size of neurons have also been described as an adaptive phenomenon due to aging in humans [36] and in animal models [44] [55]. Cytoplasmic accumulation of nuclear transport factor 2 (NTF2), a protein that transports cargo from the cytoplasm into the nucleus has been observed in a subset of hippocampal neurons in AD brains. This observation raises the possibility that changes in nuclear size could be related to abnormalities in the transport between nucleus and cytoplasm [49]. In contrast to our findings in asymptomatic AD, the MCI and AD groups show nuclear volumes comparable to those of old controls despite the presence of neuronal atrophy. This result contrasts with a previous findings of nuclear atrophy in the temporal cortex of AD cases [27]. The different outcome may be explained by examination of a different cortical region in more advanced cases of AD. The lack of nuclear enlargement suggests that neurons in MCI and AD cannot react to or have been overwhelmed by A β deposition and Tau changes, and have entered the final stretch of neuronal degeneration [13] [9].

We also observed significant atrophy of the cell body of ACG and CA1 neurons in MCI (10.1% and 9.8%, respectively) and AD (23.1% and 39.0%, respectively) compared to Controls (Fig.

2B and Fig. 4B). Our finding of neuronal atrophy in MCI is novel and its magnitude, compared to Controls and demented subjects, is consistent with other neuropathological studies that place MCI as an intermediate stage between Controls and AD [43] [35] [39]. The atrophy of the neuronal cell bodies has been previously reported in AD [46] [9] [16] in the midfrontal region [16], prefrontal area [10] and other cortical regions [9]. Our observations extend this finding to the ACG and hippocampus, suggesting that neuronal atrophy in AD is a more generalized phenomenon throughout the cerebral cortex.

The decrease in the mean volume of ACG and CA1 neurons, observed both in AD and MCI, could be attributed to generalized neuronal atrophy and/or selective loss of large neurons. The volume weighted distribution of ACG neurons reveals an expansion in the absolute volume corresponding to small neurons in the AD and MCI groups compared to Controls (data not shown). This change in distribution, which is in agreement with other authors [10], indicates that at least some neurons have shrunken, thus the increase in the total volume corresponding to small neurons. However, our analysis cannot rule out the possibility of a concomitant selective loss of larger neurons.

We believe that atrophy of the neuronal cell body reflects the shrinkage of the entire neuron, including its dendritic and axonal processes [15] in combination with loss of synapses [28], and is the cellular substrate of the cortical atrophy observed by neuroimaging or macroscopically. Although the mechanism of neuronal atrophy in AD and MCI is unknown, it is most likely mediated by changes of the neuronal cytoskeleton [41] and its constituent proteins, but the events upstream to these changes are uncertain. Possible mechanisms include loss of afferent or efferent contacts [15], neurotrophic factors [38], direct toxic effect of A β [25], or oxidative injury triggered by A β [11].

In conclusion, we have shown that in asymptomatic AD, cortical and hippocampal neurons undergo significant nuclear hypertrophy, probably as a reaction to A β , long before the onset of cognitive decline or dementia. This nuclear hypertrophy may constitute a surrogate for the activation of metabolic pathways that forestall the progression of AD and allow the brain to resist the development of dementia.

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Figure 1.

Anterior Cingulate Gyrus. **A:** Brodmann's map of the human cerebral cortex [7] showing area 24 (red) and the level where the coronal section (**B**) was taken (black line). **B:** Coronal section with highlighted anterior cingulate cortex (red) at the level of the genu of the corpus callosum depicting the manner in which the punch (white rectangle) was taken perpendicular to the surface of the gyrus. **C: Left:** Schematic representation of the punch taken demonstrating gray (red) and white matter. **Right:** Schematic representation of the half of the cylinder with the gray matter outlined (rectangle in green) showing that all the cortical layers (from the leptomenigeal surface to the white matter) were included in the contour that was analyzed. **D:** Image of a pyramidal neuron with the Vertical Nucleator probe of the system used. The red

marker is placed by the operator where the nucleolus is in focus, then the system places the rays (yellow) in a random angle, and finally the operator places the markers (green) where the rays intersect with the cell or nuclear membrane.

Figure 2. Anterior Cingulate Gyrus

A: Nuclear volumes (μm^3). **B:** Neuronal cell body volumes (μm^3). ASYMAD: asymptomatic AD. MCI: Mild Cognitive Impairment. AD: Alzheimer's Disease. Box plot: The box encloses the middle half of the values, bound by the upper and lower quartiles. The bar across the box is the mean and the dot represents the median. The lines at the end of the whiskers correspond to the largest and smallest sample values. Top p-values are in reference to Controls, bottom p-values are in reference to ASYMAD.

Figure 3. Pyramidal neurons in the Anterior Cingulate Gyrus of
A: Control, B: Asymad, C: MCI, and D: AD. (100X, Cresyl Violet)

Figure 4. Hippocampus CA1 region

A: Nuclear volumes (μm^3). **B:** Neuronal cell body volumes (μm^3). ASYMAD: asymptomatic AD; MCI: Mild Cognitive Impairment. AD: Alzheimer's Disease. Box plot: The box encloses the middle half of the values, bound by the upper and lower quartiles. The bar across the box is the mean and the dot represents the median. The lines at the end of the whiskers correspond to the largest and smallest sample values. Top p-values are in reference to Controls, bottom p-values are in reference to Asymptomatic AD.

Figure 5. Quantitation of Aβ and Tau immunoreactivity in Anterior Cingulate Gyrus

A: Aβ Fraction area $\pm$ Standard Deviations. Control: $0.132 \pm 0.25 \mu\text{m}^2$; ASYMAD: $0.478 \pm 0.33 \mu\text{m}^2$; MCI: $0.901 \pm 0.52 \mu\text{m}^2$; AD: $2.214 \pm 2.27 \mu\text{m}^2$. B: Tau-protein fraction area $\pm$ Standard Deviations. C: $0.001 \pm 0.003 \mu\text{m}^2$; ASYMAD: $0.005 \pm 0.0011 \mu\text{m}^2$; MCI: $0.023 \pm 0.041 \mu\text{m}^2$; AD: $0.213 \pm 0.25 \mu\text{m}^2$. ASYMAD: asymptomatic AD; MCI: Mild Cognitive Impairment. AD: Alzheimer's Disease. The fractional areas of both Aβ and Tau were significantly increased ($P < 0.001$) in AD compared with all other groups. There was no significant difference between ASYMAD and MCI. Y axis shows values as percentage of area. Box plot: The box encloses the middle half of the values, bound by the upper and lower quartiles. The bar across the box is the mean and the dot represents the median. The lines at the end of the whiskers correspond to the largest and smallest sample values.

Table 1
Demographic and Neuropathological Characteristics. Pathology and mean age of the sample

Age-matched Controls (CDR= 0)						Asymptomatic AD (CDR= 0)					
Subject	CERAD Score	Braak Staging	Age (years)	Subject	CERAD Score	Braak Staging	Age (years)	Subject	CERAD Score	Braak Staging	Age (years)
1 [#]	0	4	95	10 [#]	C	4	91				
2 [#]	0	4	86	11 [#]	C	3	75				
3 [#]	0	2	80	12 [#]	C	2	75				
4 [#]	0	0	57	13 [#]	B	4	92				
5	0	4	83	14 [#]	C	2	72				
6	0	0	93	15	B	4	94				
7 [#]	0	2	84	16	B	2	79				
8 [#]	0	2	81	17 [#]	B	4	93				
9	0	2	83	18 [#]	B	4	94				
Mean			82.4±10.8	Mean			85.0±9.4				

Mild Cognitive Impairment (CDR=0.5)						Alzheimer's Disease (CDR≥1)					
Subject	CERAD Score	Braak Staging	Age (years)	Subject	CERAD Score	Braak Staging	Age (years)	Subject	CERAD Score	Braak Staging	Age (years)
19 [#]	A	2	68	28	C	6	96				
20 [#]	A	3	96	29 [#]	B	4	90				
21 [#]	A	2	90	30	C	2	97				
22 [#]	B	4	99	31 [#]	C	5	80				
23 [#]	B	4	91	32	C	4	89				
24	A	2	77	33 [#]	C	6	92				
25 [#]	B	4	78	34 [#]	C	4	98				
26	B	4	89	35 [#]	C	6	89				
27	B	3	87	Mean			91.3±5.8				
Mean			86.1±9.9								

CERAD Score is for neuritic plaques, Braak Score is for neurofibrillary tangles.

; subjects whose cerebellum was studied.

Although the mean age of AD subjects was higher than in other groups, this difference was not statistically significant.

Table 2
Mean $\pm$ Standard Deviation values of nuclear and neuronal cell body volumes

	Controls (μm^3)	ASYMAD (μm^3)	MCI (μm^3)	AD (μm^3)
Anterior Cingulate Gyrus				
Nuclear volume	348.7 $\pm$ 39.6	498.1 $\pm$ 106.8	387.5 $\pm$ 25.5	303.7 $\pm$ 31.1
Cell body volume	1620.6 $\pm$ 212.2	1836.0 $\pm$ 295.6	1457.3 $\pm$ 204.4	1244.7 $\pm$ 297.5
Hippocampus CA1				
Nuclear volume	568.8 $\pm$ 146.5	791.2 $\pm$ 220.5	628.0 $\pm$ 178.0	433.3 $\pm$ 78.06
Cell body volume	3627 $\pm$ 510.1	4148 $\pm$ 831.6	3270 $\pm$ 1130.0	2209 $\pm$ 617.7
Cerebellum (Purkinje cells)				
Nuclear volume	995.1 $\pm$ 162.1	1069.5 $\pm$ 119.1	881.5 $\pm$ 247.2	1132.5 $\pm$ 103.1
Cell body volume	11803.3 $\pm$ 2893.0	12424 $\pm$ 1471.0	11924 $\pm$ 2871.0	14011.9 $\pm$ 2158.3