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Entorhinal cortex of aged subjects with Down's syndrome shows severe neuronal loss caused by neurofibrillary pathology

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Abstract In Alzheimer's disease (AD), neurofibrillary degeneration of neurons starts in the transentorhinal cortex and spreads in a time-dependent manner to the entorhinal cortex, which provides a major input to the hippocampus – a key structure of the memory system. People with Down's syndrome (DS) develop neurofibrillary changes more than 30 years earlier than those with sporadic AD. To characterize AD-related pathology in the entorhinal cortex in DS, we examined seven subjects with DS of 60–74 years of age who died in the end stage of AD, and four age-matched control subjects. The volume of the entorhinal cortex in brains of subjects with DS was 42% less than that in control cases; however, the total number of neurons free of neurofibrillary changes was reduced in DS by 90%: from 9,619,000 ± 914,000 (mean ± standard deviation) to 932,000 ± 504,000. The presence of 2,488,000 ± 544,000 neurofibrillary tangles in the entorhinal cortex of people with DS, the prevalence of end-stage tangles, and the significant negative correlation between the total number of intact neurons and the percentage of neurons with neurofibrillary changes indicate that neurofibrillary degeneration is a major cause of neuronal loss in the entorhinal cortex of people with DS. The relatively low amyloid load (7 ± 1%) and lack of correlation between the amyloid load and the volumetric or neuronal loss suggest that the contribution of β-amyloid to neuronal loss in the entorhinal cortex is unsubstantial.

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Introduction

Subjects with Down's syndrome (DS) are especially prone to develop Alzheimer-type pathology. The presence of diffuse β-amyloid (Aβ) plaques has been demonstrated in some DS subjects in the second decade of life [75], whereas the first neurofibrillary tangles (NFTs) appear in the third decade of life [68] – some 30 years earlier than in the normal population [40]. Virtually all individuals with DS older than 35 years of age develop Alzheimer-type pathology, and the numerical density of NFTs and neuritic plaques increases with age [45, 68, 78]. Neurofibrillary pathology, accumulation of Aβ, and neuronal loss result in early loss of adaptive abilities [59, 79] and lead to severe dementia [20, 73, 80], which occurs in 55% of DS subjects between 50 and 59 years of age and in 75% of DS subjects older than 60 years [42, 66].

The transentorhinal/entorhinal region is the brain area known to be affected the earliest by Alzheimer-type pathology [11]. Profound neuronal loss has been demonstrated in the entorhinal cortex (EC) in the very early stage of Alzheimer disease (AD) [25]. This loss is considered an anatomical substrate of memory and learning impairment, which is the first clinically manifested symptom of AD [17]. The EC is a mesocortical area, significantly reduced in primates and human, which serves as an interface between the association cortex and the hippocampus – the central structure in the memory system. The superficial layers of the EC receive information from many association cortical areas and relay it to the hippocampus [36, 61]. The sources of projection are mostly stellate neurons of layer II and to a lesser degree, neurons of layer III [58, 76, 77]. The hippocampus sends recurrent connections via the subicular complex to the deeper layers of the EC [6, 7, 21, 22], which relay them back to the cortex [37]. This unique position of the EC between the hip-

pocampus and the association areas make obvious why damage to the EC can result in the appearance of symptoms of memory impairment and learning disturbances [32, 33, 65].

We hypothesize that the EC in DS subjects can be affected early and severely by AD pathology, as takes place in AD patients with normal genotype. The severe damage to the EC caused by Alzheimer-type pathology could contribute significantly to the deterioration of mental status observed clinically in DS individuals during the process of aging. Because some studies indicate that in AD, neurofibrillary pathology is the major cause of neuronal loss in the hippocampus [8, 9, 16], EC [25], and temporal cortex [26], we also hypothesize that NFTs can be a major cause of neuronal degeneration in the EC in DS subjects.

The aim of this study is to characterize the type, distribution, and range of pathological changes in the entorhinal cortex of people with DS in the end stage of AD. Because in atrophic structures, relative measures such as numerical density of NFTs or neurons might be biased, the study is focused on absolute measures of the volume of the EC and the total number of neurons and NFTs.

Materials and methods

The study was performed on the EC of seven subjects with DS from 60 to 74 years of age and four age-matched control subjects (Table 1). Clinical records of all DS subjects under study showed a gradual loss of adaptive abilities over the last two decades of their life. At the time of demise, all of them were severely demented, immobilized, and bedridden and were being tube-fed. Severity of dementia was evaluated by means of Global Deterioration Scale [54] as corresponding to final stage 7. All DS cases fulfilled the clinical and postmortem criteria for diagnosis of AD. Controls had no history of long-term illness, dementia, or neurological disease, and none of these subjects had met CERAD criteria for diagnosis of AD [47].

After at least 6 weeks of fixation in 10% buffered formalin, brain hemispheres were dissected coronally into 4.8-mm-thick slabs, processed, and embedded in paraffin. Slabs were cut serially into 8-μm-thick sections. Every tenth section was stained with cresyl violet. Every 50th section was immunostained with monoclonal antibodies (mAbs) Tau-1 and 3-39 to detect neurofibrillary pathology, or with mAb 4G8 against Aβ. The Tau-1 mAb was raised against abnormally phosphorylated tau protein and detects an epitope located between amino acid residues 189 and 207 of the human tau sequence. 3-39 mAb was raised against ubiquitin and detects an epitope located between amino acid residues 50 and 65 of ubiquitin as well as epitopes on ubiquitinated paired helical filaments [5]. Immunostaining with Tau-1 was preceded by the pretreatment of sections with alkaline phosphatase (Sigma Type VII-L, 400 μg/ml in PBS, pH 7.4) at room temperature. Overnight incubation with a mixture of primary antibodies (Tau-1 1:50,000, and 3-39 1:10,000) was followed by 2-h incubation at room temperature with the secondary antibody – biotinylated anti-mouse immunoglobulin from sheep (1:200, Amersham) and 1-h incubation with extravidin-horseradish peroxidase complex (1:200, Sigma). The reaction was visualized by means of 3,3′-diaminobenzidine (DAB), and sections were counterstained with cresyl violet.

4G8 mAb was raised against the 17–24 amino acid sequence of the Aβ protein. To enhance staining, the sections were pretreated with 88% formic acid for 30 min. A 1:50,000 dilution of mAb 4G8 was used. All subsequent steps of the procedure were similar to those with staining with mAbs Tau-1 and 3-39.

In our study, we followed the pattern of EC lamination proposed by Amaral and Insausti [1], who distinguished six layers: four cellular (II, III, V, VI) and two acellular (I, IV-lamina dissecans). No divisions of the EC into subregions were taken into account. The planimetry was performed on serial sections stained with cresyl violet, 800 μm apart. Using a Documator DL-2 (Zeiss, Jena Germany), a picture of the histological specimen was enlarged 28 times, and the outlines of the EC and its layers were traced. The cross-sectional area of the EC and its layers was measured with a Sigma-Scan (Jandel Scientific Corp., San Rafael, Calif.) morphometric program, and the total volume of the structure V (mm^3) was calculated using the DeVito formula, which contains a correction factor for differences in area between two consecutive sections [18]:

$$V = \Sigma V_n$$

where V_n = the subvolume of the structure between two serial sections having areas A_n and A_n+1 (mm^2) separated by the distance Z (in our study, 0.8 mm). V_n was calculated from the following equation:

$$V_n = \frac{Z}{3} \times \left(A_n + A_{n+1} + \sqrt{A_n + A_{n+1}}\right)$$

The total numbers of intact neurons and NFTs were calculated for layers II, III, V, and VI. Five stages of NFT development were distinguished in sections stained with mAbs Tau-1 and 3-39 and counterstained with cresyl violet [5]. Pretangles (stage 0) are neurons with a scattered, granular immunopositive reaction within the perikaryon. Early tangles (stage 1) are neurons with fibrillar or rod-shaped immunopositive inclusions within a perikaryon. In im-

Table 1 Characteristics of control and DS cases (*DS* Down's syndrome)

Group	Case no.	Age (years)	Sex	Hemisphere weight (g)	Cause of death
Control	1	57	M	720	Sepsis
	2	59	F	560	Cardiac arrest
	3	61	M	710	Pneumonia
	4	68	F	625	Retroperitoneal bleeding
DS	1	60	M	430	Pneumonia
	2	62	M	515	Pulmonary edema
	3	62	F	400	Cardiac arrest
	4	63	M	420	Bronchopneumonia
	5	63	M	485	Pulmonary edema
	6	64	F	420	Cardiac arrest
	7	74	M	345	Congestive heart failure

mature tangles (stage 2), the cytoplasm is filled partially or entirely with dense immunopositive bundles, and the nucleus is shrunken and dislocated. In end-stage tangles (stage 3A), the entire perikaryon is filled with dense immunopositive material and the nucleus is not discernible. Ghost tangles (stage 3B) are extracellular bundles of loosely arranged filaments that are labeled poorly with mAb Tau-1 but strongly with 3-39. Stages 0, 1, and 2 are considered living neurons with neurofibrillary changes. Stage 3A corresponds to dead cells, whereas stage 3B represents cell remnants. The numerical density of intact neurons and of NFTs at particular stages was evaluated at × 820 magnification by means of a Pictoval projective microscope (Zeiss). The systematic random sampling scheme was applied [27, 69]. The first test area was selected, and the next areas were taken in the raster pattern from the starting point. The coefficient of error, as a measure of the sampling accuracy [25, 69], was calculated for each case and each layer separately, and it was found to be less than 0.06. The density of objects per cubic millimeter and the total number of objects were calculated according to the following equations, respectively [28]:

$$N_v = \frac{N}{(t + d - 2k) \times A}$$

where N_V = number of objects per mm^3; N = number of objects in the test area; t = section thickness (mm); d = mean diameter of object (mm); k = the correction factor (mm); and A = test area (mm^2).

$$N_t = V \times N_V$$

where N_t is the total number of objects. To calculate the density of intact neurons and neurons with NFTs per cubic millimeter, the mean diameter of nucleolus was used as the parameter d. As NFTs in stage 3A by definition do not have a discernible nucleus, and those in stage 3B are extracellularly located bunches of loosely arranged filaments, for those objects, the d value was evaluated from the tangle profile [8, 9, 16]. The d value was calculated for each case and each layer of EC separately and was on average 3.4 μm for the nucleolus and 15.7 μm for the tangle profile. The correction factor k, which adjusts N_V to compensate for those small cut cups of the objects that lie inside the section and cannot yet be discriminated visually, was calculated according to the method described by Konigsmark et al. [41]. The thickness of the section (t) was controlled by focusing a × 100 oil objective throughout the section and measuring the distance between the first and last object (nucleus of oligodendrocyte or ependymocyte) that came into focus. The measurements were made using a Digimatic Indicator IDF-150E (Mitutoyo Corp. Osaka, Japan), which registers every movement of microscope stage along the z axis with an accuracy of 0.5 μm.

The amyloid load, i.e., the percentage of EC occupied by Aβ deposits detected with mAb 4G8, was quantified using the "C IMAGING" (COMPIX, Cranberry Township, Pa.) image analysis system. A gray level threshold was set separately for any analyzed section to discriminate Aβ deposits from the background. The coefficient of error was calculated for amyloid load measurements, as it was for neuron and NFT densities, and was less than 0.06.

The shrinkage factor, a measure of distortions of the tissue that take place during histological processing, was calculated for each studied case [55]. Because the shrinkage in the histological procedure varied significantly among the brains studied (range 0.25–0.59; $P < 0.05$, analysis of variance [ANOVA]), the volume and the numerical density of objects were corrected by the shrinkage factor and presented in the corrected version, which refers to values of fixed, unprocessed tissue.

Statistical analysis was performed using the independent *t*-test and Pearson's correlation coefficient. Differences in the magnitude of the shrinkage factor among studied cases were evaluated by means of ANOVA.

Results

Volume of the EC

The decrease in the total volume of the EC from 1,330 ± 250 mm^3 (mean ± standard deviation) in the control group to 770 ± 320 mm^3 in aged subjects with DS gives a 42% volumetric loss ($P < 0.05$; Table 2). The most prominent decrease was observed in the islands of stellate neurons of layer II, where the volume was 69% smaller than that of the controls ($P < 0.01$). In DS, volumes of layers III and IV were 55% and 62%, respectively, smaller than those in controls ($P < 0.05$ and $P < 0.01$, respectively). Volumetric

Table 2 Volume of the EC and its layers (mm^3) (*EC* entorhinal cortex, *SD* standard deviation)

Group	Case no.		EC	Layer						
				I	II (total)	II (islands)	III	IV	V	VI
Control	1		1430	231	161	97	555	39	167	277
	2		1070	154	119	69	403	29	182	181
	3		1620	201	189	112	612	45	294	280
	4		1180	138	116	65	525	36	169	199
		Average	1330	181	146	86	524	37	203	234
		SD	250	43	35	23	88	7	61	51
DS	1		990	135	108	44	291	11	249	204
	2		640	67	55	17	196	11	163	149
	3		1330	165	109	46	427	21	340	268
	4		370	36	21	11	113	4	90	101
	5		860	154	108	41	249	16	165	168
	6		540	67	41	14	170	26	143	92
	7		640	79	48	13	185	10	160	154
		Average	770	100	70	27	233	14	187	162
		SD	320	50	37	16	103	8	82	60

loss in layers I and VI (44% and 31%, respectively) was less but was still significant ($P < 0.05$); however, the 7% reduction in volume of layer V in DS subjects was not significant.

Numerical density of neurons

In control cases, the numerical density of intact neurons ranged from 87.1 ± 22.7/mm^2 in layer III to 122.6 ± 18.0/mm^2 in layer II (Table 3). In DS subjects, the numerical density of neurons was significantly reduced in any layer of the EC. In four of seven examined DS cases, no intact neurons were found in layer II. In three other cases, their density ranged from only 0.1/mm^2 to 3.8/mm^2. In layers III and V, the numerical densities were 89% and 83%, respectively, fewer than those in the control group ($P < 0.01$). The least, but still severe reduction in the numerical density of intact neurons (67%) was noticed in layer VI ($P < 0.01$).

Total number of neurons and neuronal loss

In control cases, there were on average 9,619,000 ± 914,000 intact neurons in the whole EC. The sum of intact neurons and NFT-bearing neurons was calculated to be 9,647,000 ± 911,000 (Table 3). The total number of intact neurons in EC in DS subjects was on average 932,000 ± 504,000, whereas the total number of all neurons, including neurons with neurofibrillary pathology, was 1,816,000 ± 664,000. These give the difference in the total number of intact neurons, 90% and 81% in the total number of all neurons. The most remarkable reductions were noticed in layer II (99.9% of intacts and 98% of all neurons), and in layer III (96% of intact neurons and 98% of all neurons) ($P < 0.01$). In layers V and VI, the total numbers of intact neurons were 86% and 79% fewer than those in the controls, respectively, whereas the total number of all neurons was decreased by 70% and 71%, respectively ($P < 0.01$).

Numerical density and total number of NFTs

A small number of neurons with neurofibrillary changes were found in all control cases in all four cellular layers with remarkable interindividual and laminar variations. The estimated average of total number varied from 12,300 in layer II to about 600 in layer VI (Table 4).

Severe neurofibrillary pathology was found in all DS cases and in all cellular layers, and the difference in comparison to the control group was significant ($P < 0.01$). The largest numerical density of NFTs was found in layers V and II, 67.9 ± 22.7/mm^2 and 53.7 ± 19/mm^2, respectively. The NFT densities of layers III and VI were almost twofold smaller, and were calculated to be 38.6 ± 14/mm^2 and 32.5 ± 14.1/mm^2, respectively.

The total number of NFTs in the DS group, both in the whole EC (2,489,000 ± 545,000) and in particular layers, was significantly larger than that in the control group ($P < 0.01$). The largest number of NFTs was found in layers V (1,101,000 ± 270,000) and III (787,000 ± 232,000). There were 479,000 ± 209,000 NFTs in layer VI and 121,000 ± 50,000 in layer II.

Types of NFTs

In the control group, stages 0 and 1 of NFTs prevailed and constituted together 61.6% of the whole NFT population

Table 3 Numerical density and total number of intact neurons in the EC and its layers (*EC* entorhinal cortex, *SD* standard deviation)

Group	Case no.		Numerical density (N/mm^2)				Total number ($\times 10^3$)				
			II	III	V	VI	EC	II	III	V	VI
Control	1		99.8	67.9	106.0	87.2	8610	931	3640	1706	2332
	2		143.1	110.8	115.5	119.1	9380	959	4311	2025	2088
	3		127.7	67.5	113.9	81.5	10810	1385	3988	3237	2204
	4		119.6	102.3	108.3	102.7	9670	747	5187	1768	1970
		Average	122.6	87.1	110.9	97.6	9620	1006	4281	2184	2148
		SD	18.0	22.7	4.5	16.9	910	270	663	716	155
DS	1		0.0	1.3	6.2	9.2	370	0.0	37	148	181
	2		3.8	28.7	42.0	43.2	1830	6.3	543	663	621
	3		0.1	3.9	13.7	14.9	990	0.3	162	450	385
	4		0.0	2.1	18.3	24.2	420	0.0	23	160	236
	5		0.0	5.7	13.6	33.0	890	0.0	137	217	536
	6		0.0	21.0	24.9	62.4	1250	0.0	346	345	556
	7		0.1	1.2	11.3	38.8	770	0.2	22	175	578
		Average	0.6	9.1	18.6	32.2	930	1.0	181	308	442
		SD	1.4	11.1	11.9	18.1	500	2.3	197	192	176

Table 4 Numerical density and total number of NFTs in the EC and its layers (*NFTs* neurofibrillary tangles, *EC* entorhinal cortex, *SD* standard deviation)

Group	Case no.		Numerical density (N/mm^2)				Total number ($\times 10^3$)				
			II	III	V	VI	EC (total)	II	III	V	VI
Control	1		3.1	0.5	0.4	0.0	59	29	25	6	0.0
	2		0.4	0.0	0.0	0.0	3	2	0.0	0.0	0.0
	3		1.6	0.1	0.8	0.1	48	18	6	22	2
	4		0.0	0.0	0.6	0.0	9	0.0	0.0	9	0.0
		Average	1.3	0.1	0.4	0.0	30	12	8	10	0.6
		SD	1.4	0.2	0.3	0.0	28	13	12	9	1
DS	1		32.6	25.5	31.1	17.4	1950	139	718	747	342
	2		67.8	28.9	72.6	19.6	2080	111	547	1146	282
	3		36.9	23.4	45.2	33.1	3470	166	963	1487	856
	4		50.5	50.6	93.0	59.0	1990	52	553	812	575
	5		48.7	46.3	65.8	25.7	2770	193	1113	1049	418
	6		88.4	60.3	78.8	32.5	2490	120	992	1092	290
	7		51.1	34.9	88.8	40.0	2660	66	624	1376	596
		Average	53.7	38.6	67.9	32.5	2490	121	787	1101	480
		SD	19.0	14.0	22.7	14.1	540	51	232	270	209

Table 5 Staging of NFTs (%) in control and DS subjects (*NFTs* neurofibrillary tangles, *DS* Down's syndrome)

	Stage 0	Stage 1	Stage 2	Stage 3A	Stage 3B
Control	22.9	38.7	31.5	6.9	0.0
DS	1.8	4.1	29.4	41.9	22.8

Table 6 Staging of NFTs (%) per lamina in DS (*NFTs* neurofibrillary tangles, *DS* Down's syndrome)

Layer	Stage 0	Stage 1	Stage 2	Stage 3A	Stage 3B
II	0.1	0.2	11.5	45.4	42.8
III	2.8	5.0	34.0	39.7	18.5
V	1.1	2.8	26.9	39.5	29.7
VI	2.7	6.0	30.1	52.8	8.4

(Table 5). In this group, NFTs in stage 2 constituted 31.5%, but NFTs in stage 3A only 6.9%. No NFTs were observed in stage 3B in the controls. In the group of aged subjects with DS, the proportions of various stages of NFTs were reversed. Stages 0 and 1 constituted only 5.9% of the whole NFT population in this group, whereas stages 3A and 3B altogether constituted 64.7%.

There were significant differences in occurrence of various types of NFTs among particular layers in the DS group. The highest percentage of late-stage NFTs (3A and 3B) was found in layer II. They constituted together 88.2% of all NFTs in this layer, and NFTs 3B alone constituted 42.8% (Table 6). Layer V was characterized by slightly less advanced neurofibrillary pathology. NFTs in stages 3A and 3B constituted 69.2% in this layer, and NFTs in stage 3B alone, 29.7%. Layers III and VI also showed a high percentage of late-stage NFTs (58.2 and 61.2%, respectively) but the 3B NFTs constituted in these layers only 18.5 and 8.4%, respectively.

Correlations between neuronal loss and neurofibrillary pathology

The relationship between neuronal loss and neurofibrillary pathology was examined. The total number of intact neurons in the entire EC showed a strong negative correlation with the percentage of neurons involved in neurofibrillary pathology (percentage of NFTs-bearing neurons among all neurons; $r = 0.89$, $P < 0.01$).

β-Amyloid

Aβ plaques were absent in three of four control cases. In the oldest control case, amyloid occupied 3.5% of the EC. The amyloid load in DS cases was relatively small and ranged from 5.2 to 8.6% (on average 7 ± 1.1%). Most Aβ plaques were classified as either primitive or diffuse. Classical plaques occurred relatively rarely. There was no significant correlation between amyloid load and age, volumetric loss in the EC, total number of neurons, total number of intact neurons, neuronal loss, or total number of NFTs.

Discussion

The volume of the EC of subjects with DS in the end stage of AD was reduced by 42% in comparison to the age-matched control group. An almost identical volumetric loss was found in sporadic AD [25], despite significant differences in the time of onset of Alzheimer-type pathology and the age at the time of demise in the studied DS group and in sporadic AD. The volume loss in AD results directly from neuronal loss, which is an anatomical substrate of dementia. The neuronal loss in AD is known to

be both regional and laminar specific [12, 15, 31, 48]. The significant 90% deficit in the total number of neurons that was found in AD subjects with DS was larger than one could expect from 42% atrophy. The magnitude of neuronal deficit was larger than that found in the EC [25], in the hippocampal formation [8, 46, 70], and in the neocortical areas [13, 30, 46, 48, 64] in advanced AD subjects who showed a profound disturbance of memory and severe dementia. Particular layers of the EC showed various degrees of neuronal loss. Layer II was the most severely affected. In four of seven studied cases, virtually no intact neurons existed, and in the other three cases, their number ranged between 200 and 6,300. This means an almost 100% neuronal loss in this layer. Layer II is considered to be of critical importance for functioning of the memory system as neurons of this layer give rise to the perforant pathway – a major afferent pathway of the hippocampus – which reaches the outer two-thirds of the dentate gyrus molecular layer. It has been shown that a 57% loss of the neurons in layer II in the early stage of AD is associated with memory impairment [25]. Subjects in advanced stages of AD show an 87% neuronal loss in this layer, and laminar spongiosis in the molecular layer of the dentate gyrus as a result of degeneration of the perforant pathway axons [19]. However, an 87% neuronal loss is still less than that observed in DS individuals with AD. Layer III was less affected than layer II, but 97% neuronal loss also means total destruction of this layer. Neuronal deficits of 86% and 79% in layers V and VI, respectively, can be considered as slightly smaller, but they are larger than those in advanced AD cases, where layer V demonstrates 69%, and layer VI, 63% neuronal deficits, respectively [25]. Although layers III, V, and VI are considered to be of less physiological importance than layer II, their destruction also severely compromises the function of the EC as an important link between the association cortex and the hippocampus. Layer III gives rise to the perforant pathway as layer II does, and together with layers V and VI, receives back input from the hippocampus and redistributes it to the association cortex. The profound neuronal loss in the EC in AD subjects with DS indicates that the function of the EC is severely impaired, contributing to impairment of cognition and symptoms of dementia.

All analyzed DS cases showed very advanced neurofibrillary pathology. The number of tangles exceeded the number of living neurons, and most NFTs represented the end stage of NFT evolution. Among all EC layers, layer II was the most susceptible to neurofibrillary pathology. Almost 90% of NFTs in this layer were classified as either NFT 3A or NFT 3B, both of which are recognized as dead neurons. Virtually no early stages of NFTs or intact neurons occur in this layer. This suggests that in DS with AD, as in AD in non-DS, neurofibrillary pathology starts in layer II in the very early stage of disease [11] and is responsible for neuronal loss. In DS with AD subjects, layer V was the layer of the EC that was the second most affected by neurofibrillary pathology. Observations performed on AD subjects and on aged healthy individuals show that layer V follows layer II in susceptibility to neurofibrillary pathology [11]. Also, in our controls, NFTs occurred in layer V more frequently and in higher numbers than in other layers except layer II. Layers III and VI also showed very advanced neurofibrillary pathology, and the percentage of late NFT stages was higher than that in immobile patients in the end stage of AD [8]. These observations point out that the pattern of laminar susceptibility to neurofibrillary pathology in DS subjects is similar to that in normal populations, but the advancement of pathology is greater than in the end stage of AD.

Apart from NFTs, β-amyloidosis is the other hallmark of AD pathology. In DS subjects, as a result of triplication of chromosome 21, which harbors the amyloid precursor protein gene, Aβ is detectable in the brain parenchyma at as early as 21 weeks of gestation [63], and the first Aβ plaques form in the second decade of life [74, 75]. Several studies reported a very high number of Aβ plaques in various brain regions [29, 75]. Their number exceeded the number of Aβ plaques found in analogous regions in AD [29, 34]. Surprisingly, our study revealed relatively low amyloid load in the EC of DS subjects with AD. Similarly, Giannakopoulos et al. [24] did not find statistically significant differences in the numerical density of senile plaques in the EC between normal subjects with AD and non-demented age-matched controls. They also demonstrated that the density of senile plaques in the EC in AD subjects was markedly lower than that in neocortical areas.

The role of β-amyloidosis in disease progression and its impact on neuronal loss in AD are under discussion. Although both neuropathological observations and cell culture studies support a causal role of Aβ in the degeneration and death of neurons in AD [14, 23, 44, 50, 51], most morphometric studies imply that β-amyloidosis has much less impact on neuronal loss and clinical progression of the disease than neurofibrillary pathology. So far, several reports have clearly demonstrated a significant correlation between advancement of neurofibrillary pathology and neuronal loss [25, 26], atrophy of a structure [8], or duration and severity of AD [4]. All of these reports simultaneously showed a lack of correlation between the number of Aβ plaques and neuronal loss, atrophy, or severity of symptoms. However, these studies did not differentiate between diffuse and neuritic plaques and neuronal loss and dementia.

In our study, the neuronal deficit in the EC correlated with the percentage of NFT-bearing neurons, which means that the greater the number of neurons that are involved in neurofibrillary pathology, the more neuronal loss is observed. Simultaneously, amyloid load did not show correlations with age, atrophy of EC, or neuronal loss. These observations implicate neurofibrillary pathology, but not β-amyloidosis, as a major cause of neuronal loss in the EC in DS.

When the sums of total numbers of intact neurons, neurons with NFTs, and extracellular NFTs were compared, they were apparently lower in the DS group than in the control group. It ranged among studied DS from 2,320,000 to 4,460,000 compared with the range from

8,669,000 to 10,858,000 among the controls. In sporadic AD, a similar deficit was noticed in the temporal cortex by Gomez-Isla et al. [26] but not in the subiculum by Cras et al. [16]. However, Cras et al. [16] calculated and added only the numerical densities of neurons and tangles and did not take into consideration the apparent atrophy of the subiculum [8]. In contrast, our study and that of Gomez-Isla et al. studies were based on the total number of objects in total and reference volumes, respectively. This apparent deficit of number of NFTs and neurons between DS subjects with AD and the controls has three possible causes: (1) extracellular NFTs are cleared without leaving the trace, (2) some neurons in the EC die not developing NFTs, and (3) the EC has primarily trisomy 21-related neuronal deficit. The extracellular NFTs show relative insolubility and are characterized by extensive protein cross-linking [56, 57], and they are known to reside in the brain parenchyma for a long time. However, they are also infiltrated and dispersed by reactive astrocytes [52, 78] and are surrounded by activated microglial cells [49], which suggests that they are finally cleared. Some authors [16] suggest that NFTs cannot be removed, but this notion remains unfounded.

Although low amyloid load and lack of correlation with severity of neuronal loss do not favor β-amyloidosis as a major cause of neuronal loss in the EC, they also do not exclude that some neurons may die as a result of the toxic effect of fibrillar Aβ. Possibly, Aβ angiopathy may also contribute to neuronal loss; however, studies of Bobinski et al. [8] did not show a correlation between the density of affected vessels and neuronal loss.

Causal factors of neuronal death in AD other than neurofibrillary pathology and β-amyloidosis are under consideration. They include deficit of trophic factors, loss of cholinergic input from the Meynert basal nucleus complex [67, 71], and noradrenergic input from the locus coeruleus [10, 38]. However, the EC is known to be affected at the beginning of the disease process [11, 25], whereas neuronal loss in cholinergic and noradrenergic areas was demonstrated in advanced cases [10, 71]. Increased density of neurons with DNA fragmentation [2, 43] and altered expression of apoptosis-related genes [2, 3, 60] suggest that the apoptosis may be another pathological phenomenon leading to neuronal loss in AD. However, so far this theory has not been proved definitively [3]. Although all the above-mentioned factors can be considered causes of neuronal loss in AD, advanced neurofibrillary pathology is a remarkable feature of AD pathology in the EC from the beginning to the end stage of the disease [11, 35].

The third possible cause of the discrepancy in the number of intact neurons and NFTs between DS subjects with AD and the controls, is neuronal deficit related to chromosome 21 trisomy. If indeed DS subjects had a neuronal deficit in the EC initially, then in the final stage of AD, the number of remaining neurons and NFTs should be lower than the number of neurons in age-matched but genetically normal controls. Several studies of the brain cortex of DS subjects indicate regional and laminar neuronal deficit [62, 72]. Although there are no studies regarding the total number of neurons in the EC in young individuals with DS without AD, magnetic resonance imaging demonstrated that the volume of the parahippocampal gyrus, which harbors the EC, is larger than that in normal subjects [39, 53]. In contrast, image studies revealed marked atrophy of the cornu Ammonis [39, 53], for which histological investigation indicates a marked loss of neuronal density [62].

This study demonstrates a significant neuronal deficit in the EC of aged DS subjects with AD. This deficit indicates that EC function is severely compromised, which can contribute to clinically observed dementia. The high number of NFTs, the significant percentage of NFTs in their end stage, the significant correlation between advancement of neurofibrillary pathology and neuronal loss, and, in contrast, the low Aβ load and lack of correlation between the load and neuronal loss indicate that neurofibrillary pathology, and β-amyloidosis, is a major cause of neuronal loss in the EC in DS subjects with AD.

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References

1. Amaral DG, Insausti R (1990) Hippocampal formation. In: Paxinos G (ed) The human nervous system. Academic Press, San Diego, pp 711–756
2. Anderson AJ, Cummings BJ, Cotman CW (1994) Increased immunoreactivity for Jun- and Fos-related proteins in Alzheimer's disease: association with pathology. Exp Neurol 125: 286–295
3. Anderson AJ, Su JH, Cotman CW (1996) DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area, and effect of postmortem delay. J Neurosci 16: 1710–1719
4. Arriagada PV, Growdon JH, Hedley-Whyte ET, Hyman BT (1992) Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology 41: 631–639
5. Bancher C, Brunner C, Lassmann H, Budka H, Jellinger K, Wiche G, Seitelberger F, Grundke-Iqbal I, Iqbal K, Wisniewski HM (1989) Accumulation of abnormally phosphorylated tau precedes the formation of neurofibrillary tangles in Alzheimer's disease. Brain Res 477: 90–99
6. Bartesaghi R, Gessi T, Sperti L (1989) Electrophysiological analysis of the hippocampal projections to the entorhinal area. Neuroscience 30: 51–62
7. Beckstead RM (1978) Afferent connections of the entorhinal area in the rat as demonstrated by retrograde cell-labeling with horseradish peroxidase. Brain Res 152: 249–264
8. Bobinski M, Wegiel J, Wisniewski HM, Tarnawski M, Bobinski M, Reisberg B, De Leon MJ, Miller DC (1996) Neurofibrillary pathology – correlation with hippocampal formation atrophy in Alzheimer disease. Neurobiol Aging 17: 909–919
9. Bobinski M, Wegiel J, Tarnawski M, Bobinski M, Reisberg B, De Leon MJ, Miller DC, Wisniewski HM (1997) Relationships between regional neuronal loss and neurofibrillary changes in the hippocampal formation and duration and severity of Alzheimer disease. J Neuropathol Exp Neurol 56: 414–420

10. Bondareff W, Mountjoy CQ, Roth M (1982) Loss of neurons of origin of the adrenergic projection to cerebral cortex (nucleus locus ceruleus) in senile dementia. Neurology 32:164–168
11. Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 82:239–259
12. Braak H, Braak E (1994) Morphological criteria for recognition of Alzheimer's disease and the distribution pattern of cortical changes related to this disorder. Neurobiol Aging 15:355–356
13. Brun A, Englund E (1981) Regional pattern of degeneration in Alzheimer's disease: neuronal loss and histopathological grading. Histopathology 5:549–564
14. Busciglio J, Lorenzo A, Yankner BA (1992) Methodological variables in the assessment of beta amyloid neurotoxicity. Neurobiol Aging 13:609–612
15. Coleman PD, Flood DG (1987) Neuron numbers and dendritic extent in normal aging and Alzheimer's disease. Neurobiol Aging 8:521–545
16. Cras P, Smith MA, Richey PL, Siedlak SL, Mulvihill P, Perry G (1995) Extracellular neurofibrillary tangles reflect neuronal loss and provide further evidence of extensive protein cross-linking in Alzheimer disease. Acta Neuropathol 89:291–295
17. Cummings JL (1995) Dementia: the failing brain. Lancet 345:1481–1484
18. DeVito JL, Graham J, Sackett GP (1989) Volumetric growth of the major brain divisions in fetal Macaca nemestrina. J Hirnforsch 30:479–487
19. Duyckaerts C, Colle MA, Seilhean D, Hauw JJ (1998) Laminar spongiosis of the dentate gyrus: a sign of disconnection, present in cases of severe Alzheimer's disease. Acta Neuropathol 95:413–420
20. Evenhuis HM (1990) The natural history of dementia in-Down's syndrome. Arch Neurol 47:263–267
21. Finch DM, Nowlin NL, Babb TL (1983) Demonstration of axonal projections of neurons in the rat hippocampus and subiculum by intracellular injection of HRP. Brain Res 271:201–216
22. Finch DM, Wong EE, Derian EL, Babb TL (1986) Neurophysiology of limbic system pathways in the rat: projections from the subicular complex and hippocampus to the entorhinal cortex. Brain Res 397:205–213
23. Forloni G, Chiesa R, Smiroldo S, Verga L, Salmona M, Tagliavini F, Angeretti N (1993) Apoptosis mediated neurotoxicity induced by chronic application of beta amyloid fragment 25–35. Neuroreport 4:523–526
24. Giannakopoulos P, Hof PR, Kovari E, Vallet PG, Herrmann FR, Bouras C (1996) Distinct patterns of neuronal loss and Alzheimer's disease lesion distribution in elderly individuals older than 90 years. J Neuropathol Exp Neurol 55:1210–1220
25. Gomez-Isla T, Price JL, McKeel DW Jr, Morris JC, Growdon JH, Hyman BT (1996) Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. J Neurosci 16:4491–4500
26. Gomez-Isla T, Hollister R, West H, Mui S, Growdon JH, Petersen RC, Parisi JE, Hyman BT (1997) Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. Ann Neurol 41:17–24
27. Gundersen HJ, Jensen EB (1987) The efficiency of systematic sampling in stereology and its prediction. J Microsc 147:229–263
28. Haug H (1971) Stereological methods in the analysis of neuronal parameters in the central nervous system. J Microsc 95:165–180
29. Hof PR, Bouras C, Perl DP, Sparks DL, Mehta N, Morrison JH (1995) Age-related distribution of neuropathologic changes in the cerebral cortex of patients with Down's syndrome. Quantitative regional analysis and comparison with Alzheimer's disease. Arch Neurol 52:379–391
30. Hubbard BM, Anderson JM (1985) Age-related variations in the neuron content of the cerebral cortex in senile dementia of Alzheimer type. Neuropathol Appl Neurobiol 11:369–382
31. Hyman BT, Gomez-Isla T (1994) Alzheimer's disease is a laminar, regional, and neural system specific disease, not a global brain disease. Neurobiol Aging 15:353–354
32. Hyman BT, Van Hoesen GW, Damasio AR, Barnes CL (1984) Alzheimer's disease: cell-specific pathology isolates the hippocampal formation. Science 225:1168–1170
33. Hyman BT, Van Hoesen GW, Kromer LJ, Damasio AR (1986) Perforant pathway changes and the memory impairment of Alzheimer's disease. Ann Neurol 20:472–481
34. Hyman BT, Marzloff K, Arriagada PV (1993) The lack of accumulation of senile plaques or amyloid burden in Alzheimer's disease suggests a dynamic balance between amyloid deposition and resolution. J Neuropathol Exp Neurol 52:594–600
35. Hyman BT, West HL, Rebeck GW, Lai F, Mann DM (1995) Neuropathological changes in Down's syndrome hippocampal formation. Effect of age and apolipoprotein E genotype. Arch Neurol 52:373–378
36. Insausti R, Amaral DG, Cowan WM (1987) The entorhinal cortex of the monkey. II. Cortical afferents. J Comp Neurol 264:356–395
37. Insausti R, Herrero MT, Witter MP (1997) Entorhinal cortex of the rat: cytoarchitectonic subdivisions and the origin and distribution of cortical efferents. Hippocampus 7:146–183
38. Iversen LL, Rossor MN, Reynolds GP, Hills R, Roth M, Mountjoy CQ, Foote SL, Morrison JH, Bloom FE (1983) Loss of pigmented dopamine-beta-hydroxylase positive cells from locus coeruleus in senile dementia of Alzheimer's type. Neurosci Lett 39:95–100
39. Kesslak JP, Nagata SF, Lott I, Nalcioglu O (1994) Magnetic resonance imaging analysis of age-related changes in the brains of individuals with Down's syndrome. Neurology 44:1039–1045
40. Khachaturian ZS (1985) Diagnosis of Alzheimer's disease. Arch Neurol 42:1097–1105
41. Konigsmark BW, Kalyanaraman UP, Corey P, Murphy EA (1969) An evaluation of techniques in neuronal population estimates: the sixth nerve nucleus. Johns Hopkins Med J 125:146–158
42. Lai F, Williams RS (1989) A prospective study of Alzheimer disease in Down syndrome. Arch Neurol 46:849–853
43. Lassmann H, Bancher C, Breitschopf H, Wegiel J, Bobinski M, Jellinger K, Wisniewski HM (1995) Cell death in Alzheimer's disease evaluated by DNA fragmentation in situ. Acta Neuropathol 89:35–41
44. Lorenzo A, Yankner BA (1994) Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. Proc Natl Acad Sci USA 91:12243–12247
45. Malamud N (1972) Neuropathology of organic brain syndromes associated with aging. In: Gaitz CM (ed) Aging and the brain. Plenum Press, New York, pp 63–67
46. Mann DM, Yates PO, Marcyniuk B (1985) Some morphometric observations on the cerebral cortex and hippocampus in presenile Alzheimer's disease, senile dementia of Alzheimer type and Down's syndrome in middle age. J Neurol Sci 69:139–159
47. Mirra SS, Hart M, Terry RD (1993) Making the diagnosis of Alzheimer's disease. A primer for practicing pathologists. Arch Pathol Lab Med 117:132–144
48. Mountjoy CQ, Roth M, Evans NJ, Evans HM (1983) Cortical neuronal counts in normal elderly controls and demented patients. Neurobiol Aging 4:1–11
49. Perlmutter LS, Scott SA, Barron E, Chui HC (1992) MHC class II-positive microglia in human brain: association with Alzheimer lesions. J Neurosci Res 33:549–558
50. Pike CJ, Burdick D, Walencewicz AJ, Glabe CG, Cotman CW (1993) Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. J Neurosci 13:1676–1687
51. Pike CJ, Walencewicz-Wasserman AJ, Kosmoski J, Cribbs DH, Glabe CG, Cotman CW (1995) Structure-activity analyses of beta-amyloid peptides: contributions of the beta 25–35 region to aggregation and neurotoxicity. J Neurochem 64:253–265
52. Probst A, Ulrich J, Heitz PU (1982) Senile dementia of Alzheimer type: astroglial reaction to extracellular neurofibrillary tangles in the hippocampus. An immunocytochemical and electron microscopic study. Acta Neuropathol (Berl) 57:75–79

53. Raz N, Torres IJ, Briggs SD, Spencer WD, Thornton AE, Loken WJ, Gunning FM, McQuain JD, Driesen NR, Acker JD (1995) Selective neuroanatomic abnormalities in Down's syndrome and their cognitive correlates: evidence from MRI morphometry. Neurology 45:356–366
54. Reisberg B, Ferris SH, De Leon MJ, Crook T (1982) The Global Deterioration Scale for assessment of primary degenerative dementia. Am J Psychiatry 139:1136–1139
55. Sadowski M, Morys J, Berdel B, Maciejewska B (1995) Influence of fixation and histological procedure on the morphometric parameters of neuronal cells. Folia Morphol (Warsz) 54: 219–226
56. Smith MA, Taneda S, Richey PL, Miyata S, Yan S-D, Stern D, Sayre LM, Monnier VM, Perry G (1994) Immunocytochemical demonstration of advanced Maillard reaction products in Alzheimer's disease pathology. Proc Natl Acad Sci USA 91: 5710–5714
57. Smith MA, Richey PL, Kutty RK, Taneda S, Monnier VM, Sayre LM, Perry G (1994) Advanced Maillard reaction end products, free radicals and protein oxidation in Alzheimer's disease. Ann N Y Acad Sci 738:447–454
58. Steward O, Scoville SA (1976) Cells of origin of entorhinal cortical afferents to the hippocampus and fascia dentata of the rat. J Comp Neurol 169:347–370
59. Strauss D, Zigman WB (1996) Behavioral capabilities and mortality risk in adults with and without Down syndrome. Am J Ment Retard 101:269–281
60. Su JH, Deng G, Cotman CW (1997) Bax protein expression is increased in Alzheimer's brain: correlations with DNA damage, Bcl-2 expression, and brain pathology. J Neuropathol Exp Neurol 56:86–93
61. Suzuki WA, Amaral DG (1994) Perirhinal and parahippocampal cortices of the macaque monkey: cortical afferents. J Comp Neurol 350:497–533
62. Sylvester PE (1983) The hippocampus in Down's syndrome. J Ment Defic Res 27:227–236
63. Teller JK, Russo C, DeBusk LM, Angelini G, Zaccheo D, Dagna-Bricarelli F, Scartezzini P, Bertolini S, Mann DM, Tabaton M, Gambetti P (1996) Presence of soluble amyloid beta-peptide precedes amyloid plaque formation in Down's syndrome. Nat Med 2:93–95
64. Terry RD, Peck A, DeTeresa R, Schechter R, Horoupian DS (1981) Some morphometric aspects of the brain in senile dementia of the Alzheimer type. Ann Neurol 10:184–192
65. Van Hoesen GW, Damasio AR (1987) Neural correlates of cognitive impairment in Alzheimer's disease. In: Plum F (ed) Handbook of physiology, 1st edn. American Physiological Society, Washington, DC, pp 871–898
66. Visser FE, Aldenkamp AP, Huffelen AC van, Kuilman M, Overweg J, Wijk J van (1997) Prospective study of the prevalence of Alzheimer-type dementia in institutionalized individuals with Down syndrome. Am J Ment Retard 101:400–412
67. Vogel OJ, Broere CA, Laak HJ, Donkelaar HJ, Nieuwenhuys R, Schulte BP (1990) Cell loss and shrinkage in the nucleus basalis Meynert complex in Alzheimer's disease. Neurobiol Aging 11:3–13
68. Wegiel J, Wisniewski HM, Dziewiatkowski J, Popovitch ER, Tarnawski M (1996) Differential susceptibility to neurofibrillary pathology among patients with Down syndrome. Dementia 7:135–141
69. West MJ, Gundersen HJ (1990) Unbiased stereological estimation of the number of neurons in the human hippocampus. J Comp Neurol 296:1–22
70. West MJ, Colemann PD, Flod DG, Troncoso JC (1994) Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease. Lancet 344:769–772
71. Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237–1239
72. Wisniewski KE, Laure-Kamionowska M, Wisniewski HM (1984) Evidence of arrest of neurogenesis and synaptogenesis in brains of patients with Down's syndrome. N Engl J Med 311: 1187–1188
73. Wisniewski KE, Dalton AJ, McLachlan C, Wen GY, Wisniewski HM (1985) Alzheimer's disease in Down's syndrome: clinicopathologic studies. Neurology 35:957–961
74. Wisniewski KE, Wisniewski HM, Wen GY (1985) Occurrence of neuropathological changes and dementia of Alzheimer's disease in Down's syndrome. Ann Neurol 17:278–282
75. Wisniewski HM, Wegiel J, Popovitch ER (1994) Age-associated development of diffuse and thioflavin-S-positive plaques in Down syndrome. Dev Brain Dysfunct 7:330–339
76. Witter MP, Amaral DG (1991) Entorhinal cortex of the monkey. V. Projections to the dentate gyrus, hippocampus, and subicular complex. J Comp Neurol 307:437–459
77. Witter MP, Van Hoesen GW, Amaral DG (1989) Topographical organization of the entorhinal projection to the dentate gyrus of the monkey. J Neurosci 9:216–228
78. Yamaguchi H, Morimatsu M, Hirai S, Takahashi K (1987) Alzheimer's neurofibrillary tangles are penetrated by astroglial processes and appear eosinophilic in their final stages. Acta Neuropathol (Berl) 72:214–217
79. Zigman WB, Schupf N, Lubin RA, Silverman WP (1987) Premature regression of adults with Down syndrome. Am J Ment Defic 92:161–168
80. Zigman WB, Schupf N, Sersen E, Silverman W (1996) Prevalence of dementia in adults with and without Down syndrome. Am J Ment Retard 100:403–412