ChAT-immunoreactive neurons in the Ch1 region tend to be ovoid with vertical orientation, while most of those in the Ch2 region are fusiform-to-oval. In addition, the ChAT-immunoreactive neurons appeared to be sparser in the Ch1–2 regions of the DLB brains than in those of the AD and control brains (Fig. 1a–c). On the other hand, the degree of immunostaining was variable, ranging from light to strong in all cases (Fig. 1d–f).

When Alzheimer pathology was examined according to Braak staging [4], the control cases and most of the DLB cases were found to be in Braak stages 1–3 (Table 1). These findings did not fulfill the neuropathologic criteria for definite AD. Moreover, neurofibrillary tangles were observed in the Ch2 region of all the AD cases by using the Gallyas–Braak staining method, but were not found in the Ch2 region of the DLB and control cases (Fig. 1g). On the other hand, Lewy bodies were observed in the Ch1–2 regions of the DLB group (Fig. 1h, i).

ChAT-immunostaining revealed cholinergic neuronal changes in the Ch1–2 regions of the DLB brains. Significant differences among the three groups were found in both the number and the mean surface area of the ChAT-immunoreactive neurons projecting to the hippocampus (each $p < 0.05$, Kruskal–Wallis test). The number of these neurons was significantly reduced in the DLB group compared with the AD and control groups (each $p < 0.05$, Mann–Whitney U-test). No significant difference was found in the number of the ChAT-immunoreactive neurons between the AD and control groups, as expected from the previous studies [21, 23] (Fig. 2). On the other hand, when densitometric analysis of ChAT-immunoreactive neurons was performed, the mean surface areas of the AD and DLB groups were significantly reduced compared with the control group (each $p < 0.05$, Mann–Whitney U-test). No significant difference was found in the mean surface area of the ChAT-immunoreactive neurons between the AD and DLB groups (Fig. 3).

Discussion

In this study, we found that the cholinergic neurons projecting to the hippocampus were notably degenerated in the DLB group compared with the AD and control groups. However, no significant difference was found in the number of the ChAT-immunoreactive neurons between the AD and control groups, in agreement with the previous reports [21, 23]. Moreover, the mean surface area of ChAT-immunoreactive neurons in the AD and DLB groups was significantly reduced as compared with that in the control group. Based on the fact that atrophy of basal forebrain neurons occurs with the degenerative processes in AD [25, 29], our study showed that cholinergic neurons in the Ch1–2 regions of the AD group were relatively spared in comparison with those of the DLB group. Curiously, a previous volumetry study based on MR imaging supports our results: Brenneis et al. reported that the atrophy of the basal forebrain, including the Ch1–2 regions, of patients with DLB was significantly greater than that of patients with AD [3]. Because Lewy pathology, but not AD pathology, was observed in the Ch1–2 regions of all the DLB cases in the present study, cholinergic neuronal degeneration in the Ch1–2 regions might be specific to the pathogenesis of DLB.

The major cholinergic input to the hippocampus enters the structure via the fimbria-fornix, and the distribution of cholinergic fibers and terminals in the

Table 1 Clinical and pathological data of our cases

	Age (years)	Sex	Brain wt. (g)	Braak stage (NFT)	Duration of Illness	Cause of death
Control (non-demented)						
1	87	F	1015	2	–	Pancreas cancer
2	80	M	1260	1	–	Prostate cancer, heart failure
3	83	F	1160	2	–	Acute cardiomyocarditis
Alzheimer's disease (*AD*)						
4	84	M	1140	5	3	Pneumonia
5	83	F	1040	6	6	Gastric cancer
6	84	F	1070	5	15	Gastric cancer
7	83	M	1110	5	7	Pneumonia
Dementia with Lewy bodies (*DLB*)						
8	47	F	1060	0[a]	4	Heart failure
9	82	M	1150	3	3	Pneumonia
10	79	M	1300	3	3	Pneumonia
11	69	M	1240	3	3	Pneumonia
12	85	F	1010	3	9	Heart failure
13	72	M	1350	1	4	Pneumonia
14	84	M	1200	2	7	Pneumonia
15	82	F	1290	2	4	Pneumonia

[a]No NFT in the brain

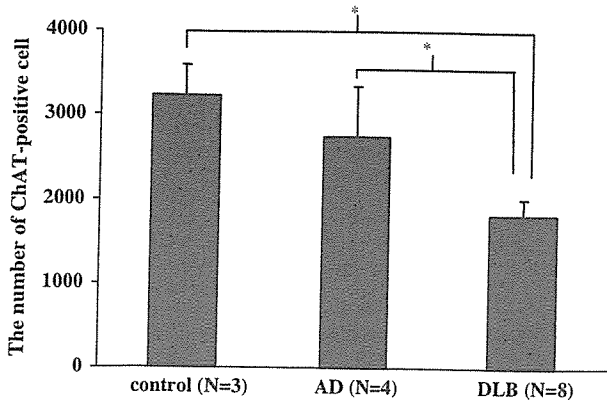

Fig. 2 The number of ChAT-positive neurons projecting to the hippocampus in each group. $*p < 0.05$

hippocampus has been reported [14]. Curiously, a previous study showed that punctate ChAT-immunostaining was conspicuous in the stratum pyramidale of the CA2–3 area, and that the highest density of ChAT-positive terminals was found in the stratum pyramidale and the juxtrapyramidal zone of the stratum oriens [27]. Another immunostaining study revealed that nerve growth factor (NGF), which plays an important role in sustaining and surviving cholinergic neurons, is mainly localized within the CA2–4 area, but is not present in the CA1 or subiculum [24]. On the other hand, Lewy-related neurites in the DLB brains are specific to the CA2–3 area in the hippocampus. In addition, ultrastructural and immunohistochemical studies suggest that these neurites are abnormal axon terminals. These findings lead us to conjecture that the depletion of cholinergic neurons projecting to the hippocampus in the DLB brain can be attributed to the Lewy-related neurites of the CA2–3 area. However, we did not investigate the ChAT-immunoreactivity of Lewy-related neurites in the present study, and thus, further studies will be needed to clarify the origin of Lewy-related neurites in the CA2–3.

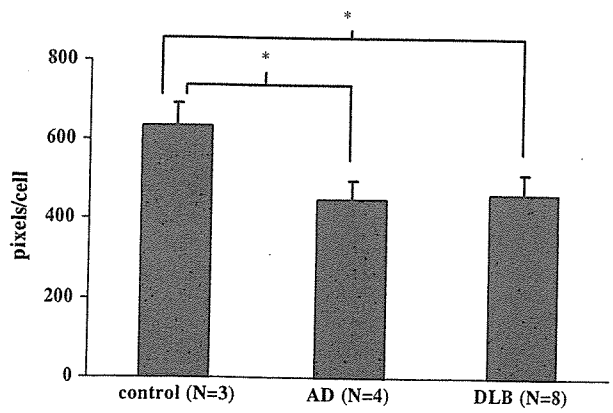

Fig. 3 The mean surface area (pixels/cell) of ChAT-positive neurons in each group. $*p < 0.05$

There have been several animal studies on immunotoxic lesions that specifically damage the cholinergic neurons projecting to the hippocampus. For example, monkeys with damages to the cholinergic neurons projecting to the hippocampus showed impaired performance on visual-spatial conditional learning tasks [28]. Moreover, rats with similar damages to cholinergic neurons showed impaired performance on environment-spatial conditional learning tasks [12]. These results may indicate that cholinergic neurons projecting to the hippocampus participate in certain visual-spatial cognitive impairments specific to DLB patients [5, 20].

Some clinical trials have reported that cholinesterase inhibitors are more effective in DLB patients than in AD patients [13, 30]. Treatment with these agents has positive effects on cognitive impairments, psychiatric symptoms, and global dysfunction. Visual hallucination, which is one of the three core clinical features in DLB, is also improved by cholinesterase inhibitors. From a clinical point of view, the severe depletion of cholinergic neurons projecting to the hippocampus in DLB might provide grounds for pharmacological intervention with cholinesterase inhibitors.

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Influence of behavioral and psychological symptoms of dementia (BPSD) and environment of care on caregivers' burden

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Abstract

With increasing population of older adults in need of care, caregiver's burden is becoming a major concern. We investigated the relative contributions of BPSD of care recipients, caregiver's background and the care environment to caregiver's burden assessed by using Zarit burden interview (ZBI). Among BPSD, inability of finding the way home, inability of managing money and fecal incontinence were the most difficult symptoms to cope with. A path analysis, by which we constructed a network model to clarify the contributions of the factors examined to the caregiver's burden, indicated that the severity of dementia, the feeling of "would rather die than be in the same condition" and the physical pain of the caregivers showed great direct influences on the score of the ZBI. In conclusion, we clarified kinetic and dynamic interactions of factors affecting caregiver's burden by using a path analysis. The model indicates that the caregiver's burden can be affected not merely by the illness of the care recipients but by the caregiver's background and the care environment.

Keywords: Care burden; Path analysis; Behavioral and psychological symptoms of dementia (BPSD)

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1. Introduction

The proportion of the population made up of individuals aged 65 and older in Japan exceeded 19.0% in 2003 (MHLW, 2003). Among the elderly population, 13% were estimated to be in need of care due to their physical or mental disabilities (MHLW, 2000). According to the MHLW (2001), 71.1% of elderly care recipients who live at home are receiving care primarily from their family members. The Japanese government launched public long-term care insurance in the year 2000, with an aim of providing care recipients with the relevant care services according to their level of disability. Because the present system for evaluating the level of disability is still in its developmental stage, further revisions are required with particular reference to the adequate assessment of dementia and related behavioral disturbances, which must be reflected in the evaluation of relevant care needs. Previous reports have demonstrated an association between the caregiver's burden and both the BPSD and the care environment, but how these factors contribute to the increase of the caregiver's burden remains to be clarified. Because the caregiver's burden is a multi-layered phenomenon involving various factors on both sides (care recipients and caregivers), clarification of the complicated relationship between these factors and the caregiver's burden should lead to a better understanding of how the burden increases, and thus of what interventions might help to reduce it.

In this study, in an attempt to clarify the structure of the caregiver's burden and how it develops, we here applied a network model using path analysis.

2. Subjects and methods

2.1. Subjects

A total of 116 caregivers of elderly patients were enrolled in this study. All patients were care recipients who either attended the geriatric outpatient of the Nagoya University Hospital or used in-home care services from community service providers. Written informed consent was obtained from all participants.

2.2. Measurements

A structured questionnaire was handed or sent to the caregivers of the care recipients. The questionnaire asked about the care recipient's and caregiver's background, clinical conditions, care environment, familial and economic status, and the caregiver's burden was sent to each of the caregivers. The severities of physical disability and dementia were evaluated according to the criteria shown in Tables 1 and 2. These criteria are normally used for evaluating the level of disability when care recipients apply for services provided for by the public long-term care insurance policy. Respondents were also queried in regard to the types of services provided and the presence or absence of an intimate counselor and an alternative caregiver. In addition, the caregivers were asked whether or not they had to relocate in order to provide care, whether the demands of providing care had forced them to

Table 1
Criteria of the severity of physical disability

1	Almost independent in daily living despite some disabilities and able to go out of home by self, going out of home by self using public transportation
2	Almost independent in daily living despite some disabilities and able to go out of home by self, going out of home by self within neighborhood
3	Almost independent in domestic daily living but unable to go out of home without assistance, spending most of day time out of bed and able to go out of home with assistance
4	Almost independent in domestic daily living but unable to go out of home without assistance, spending a considerable day time in bed and seldom go out of home
5	Spending most of day time in bed and require any assistance in daily activities but able to keep sitting position, able to move to a wheel chair from bed and do eating and toiletting out of bed
6	Spending most of day time in bed and require any assistance in daily activities but able to keep sitting position, require assistance to move to a wheel chair
7	Bed ridden all the time and require assistance for toiletting, eating and dressing, unable to roll over without assistance
8	Bed ridden all the time and require assistance for toiletting, eating and dressing, unable to roll over without assistance

quit their job, whether they found their role rewarding, and whether they ever took time off from providing care.

The BPSD of the patients were assessed using an original list (Table 3). The lists consisted of 18 symptoms (nos. 1–18) included in the primary assessment dataset of the public long-term care insurance and 17 symptoms (nos. 19–35) selected from the lists applied in previous studies (Sanford, 1975; Greene et al., 1982; Baumgarten et al., 1990). For each applicable symptom, the caregivers were asked to rate the degree of difficulty in coping with the symptom by providing a score ranging from 0 (none) to 10 (very severe).

The caregiver's burden was assessed by the Zarit burden interview (ZBI) (Zarit et al., 1980). The ZBI has 21 questions with four choices for each item, and the total score (full score: 84) was used for the analyses. We also asked the caregivers to self-rate their overall sense of burden and life satisfaction on a scale of 0 (extremely low) to 100 (extremely high).

Table 2
Criteria of the severity of dementia

0	Not demented
1	Almost independent both domestically and socially despite some dementia symptoms
2	Hampered in daily living with mental symptoms, abnormal behaviors and communication disorders, but barely maintain independence with close supervision by others
3	In constant need for assistance because of incapacity due to mental symptoms, abnormal behaviors and communication disorders. Problematic symptomes or behaviors are observed
4	Incapacitated in daily living with frequent mental symptoms, abnormal behaviors and communication disorders, and unable to maintain independence without assistance by others
5	In need of specialized medical care because of extreme mental disorders, problematic behaviors or severe physical ailments persistent manifestation of psychiatric symptomes such as delirium, delusion, agitation, or self-inflicting injury

Table 3
The list of BPSD

1	Delusion of being robbed
2	Confabulation or spread around
3	Hallucination
4	Changeable mood
5	Sleep disturbance
6	Verbal and non-verbal abuse
7	Repeated story
8	Loud voice
9	Resistant to care
10	Wandering
11	Restlessness
12	Inability in finding the way home
13	Request to go home
14	Hording useless things
15	Inability of managing the hot things
16	Destroying property
17	Filthy behavior
18	Allotriophagy
19	Confusion between present and past
20	Misrecognition for family
21	Misrecognition of acquaintance
22	Inability of managing money
23	Inappropriate sexual behavior
24	Hiding things
25	Compulsive behavior
26	Misinterpretation for caregivers' contact
27	Hanging around persistently, repetitive question
28	Disturbing conversation
29	Waking caregiver up
30	In need to be watched out
31	Reduction of interest
32	Appears unhappy or depressed
33	Abnormal appetite
34	Urinary incontinence
35	Fecal incontinence

In addition to the above assessment of caregiver's burden, with an aim to clarify relative contributions of caregiver's feeling to the burden, two questions asking a sense of loss as the care recipient's cognition declines and whether or not he/she would rather die than be in the same condition were included in the questionnaire. Also, a covert wish if the care recipient would disappear and a latent desire for dying to escape from the burden were asked.

2.3. Statistics

Pearson's correlation coefficients (r) were calculated for parametric data and Spearman's rank of order correlation coefficients (ρ) were calculated for non-parametric data. We used the chi-square test with Yates correction, and Fisher's exact test for

categorical comparisons of the data. Differences in the means of continuous measurements among the groups were tested using the Student's *t*-test and one-way analysis of variance (ANOVA). Tukey's test was performed for multiple comparisons when ANOVA showed a significant difference. The internal consistency of the ZBI was calculated by Cronbach's alpha. Multiple regression analysis, using the step-wise method with the variables of significant measures detected in the univariate analyses, was conducted to identify the factors contributing to the ZBI. Patients whose relevant data was missing were excluded from the multivariate analysis. To clarify the process by which the caregiver's burden develops, a path analysis was performed for the variables which had a significant relationship with the ZBI, using multiple regression method as described by Munro (2001) and Polit (1996). Path analysis is an extension of the regression model, used to test the fitness of the correlation matrix. A p-value of <0.05 was considered to indicate statistical significance; all tests were two-tailed. All statistical analyses were performed on a personal computer with the statistical package SPSS 11.0 and Amos 4.02 for Windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Attributes of the care recipients

Seventy-four percent of the care recipients were female. The mean age of the recipients was 79.8 ± 9.1 years (here and in all other cases ± S.D.), and the mean duration of receiving care was 46.6 ± 42.1 months. The numbers of cohabitants were: none (6.4%), one (14.7%) and more than two (78.9%). The mean severity of physical disability was 3.2 ± 2.0, and the mean severity of dementia was 2.0 ± 1.3. A majority (94.4%) of all the care recipients surveyed used some care services as follows: day service (73.8%), respite care (25.2%), home visit care (25.2%), use of supporting instruments of care (21.5%), home visit nursing (10.3%), home visit by physician (8.4%), bathing service (3.7%), home visit dentistry (2.6%) and in-home rehabilitation (0.9%).

3.2. Attributes of the caregivers and the care environments

A majority (84%) of the caregivers were female. The caregivers' relationships to the care recipients were: spouse (30.4%), daughter (30.4%), daughter-in-law (27.8%), son (8.7%) and others (2.6%). The mean age of the caregivers was 60.8 ± 11.5 months and the duration of providing care was 45.3 ± 42.2 months. A majority (79.3%) of the caregivers experienced some physical pain of their own, and many of them had to either retire from their work (19.5%) or change their residence for care (13.2%). Meanwhile, 27.2% of the caregivers answered "poor" or "mildly poor" to the question about a premorbid interpersonal relationship with the care recipients. Seventy-eight percent of caregivers had an intimate counselor, and 56% of them had an alternative caregiver. The caregivers found their roles rewarding at the following rates: always, 4.5%; often, 4.5%; sometimes, 28.2%; rarely, 24.5%; none, 38.2%. The frequencies of physical pain felt by the caregivers were: always, 22.1%; often, 8.0%; sometimes, 30.1%; rarely, 18.6%; never, 21.2%. The

frequency of respite from the care was: more than once a week, 41.1%; a few times per month, 23.4%; never, 35.5%.

Twenty-five percent of the caregivers had a strong sense of loss due to the deterioration of cognitive function of the care recipient, and 46.0% of them thought they would rather die if they were in the same status as the care recipient.

3.3. The BPSD

Fig. 1 shows the frequencies of observed BPSD and the difficulties in coping with the BPSD. The mean number of BPSD reported by the caregivers was 11.7 ± 7.9. The mean of the cumulative score of difficulties was 63.5 ± 52.1.

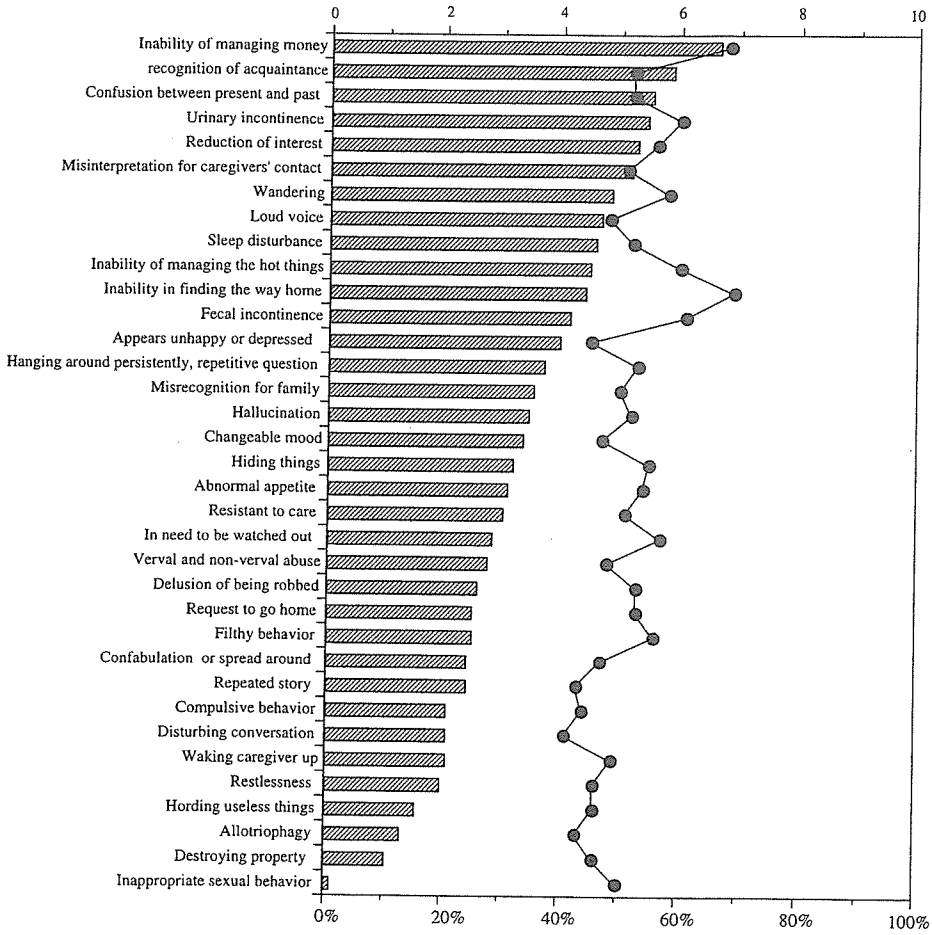

Fig. 1. The frequency of BPSD to be observed, and the difficulties of coping with each symptom. The horizontal bar chart shows the frequencies of BPSD to be observed, and the kinked line shows the difficulties to cope with the symptoms.

3.4. Caregivers' burden

The mean ZBI score was 35.3 ± 15.6. The reliability was high, with a Cronbach's α of 0.915, and the alpha was also high ($\alpha = 0.918$) when it was calculated only for the care recipients with dementia. The overall burden score was 56.6 ± 25.8, and the score was strongly correlated with the ZBI score ($r = 0.623$, $p < 0.001$). When the subjects were limited to the care recipients with dementia, Cronbach's α of the ZBI score remained high, with $\alpha = 0.918$, and the ZBI score was also strongly correlated with the burden score with an r of 0.528 ($p < 0.001$).

The severity of dementia was related with the ZBI ($r = 0.334$, $p = 0.003$), but it did not have a significant association with the burden score ($r = 0.154$, $p = 0.163$). Meanwhile, the severity of physical disability showed no significant correlation with the ZBI or the burden score.

The ZBI and the burden score were significantly associated with the duration of care (ZBI: $r = 0.223$, $p = 0.021$; burden score: $r = 0.219$, $p = 0.018$, respectively), the presence of an intimate counselor ($t = -3.685$, $p < 0.001$; $t = -2.179$, $p = 0.024$), and the frequency of physical pain in the caregivers ($\rho = -0.311$, $p = 0.001$; $\rho = 0.293$, $p = 0.002$). The presence of an alternative caregiver made the burden score slightly higher ($t = -1.988$, $p = 0.049$), but did not affect the ZBI ($t = -1.581$, $p = 0.117$). No significant difference of the ZBI or the burden score was seen depending on the caregiver's gender, age or economic status. There was no significant difference of the ZBI or the burden score depending on a familial relationship. The ZBI and the burden score were the highest if the caregiver was daughter-in-law (mean ZBI score: 38.1 ± 13.8, mean burden score: 65.0 ± 23.6, respectively). The premorbid interpersonal relationship between the caregiver and the care recipient was not significantly related with the ZBI score ($\rho = 0.034$; $p = 0.730$) or the burden score ($\rho = -0.170$; $p = 0.071$).

Multiple regression analysis for the ZBI showed that the significant variables were the severity of dementia (standardized $\beta = 0.740$), the presence of an intimate counselor (standardized $\beta = 0.289$), and the BPSD of disturbing conversation (standardized $\beta = 0.294$), appears unhappy or depressed (standardized $\beta = 0.304$) and urinary incontinence (standardized $\beta = 0.205$) with an adjusted R^2 of 0.401.

3.5. The influence of burden on the caregivers' mental status

The covert wish if the care recipient should disappear was observed consistently in 5.2% of the caregivers, often in 1.7% of them, sometimes in 17.2%, occasionally in 31.0%, and 44.8% of the caregivers never had the wish. The wish was highly related with the ZBI and the burden score (ZBI: $\rho = 0.431$, $p < 0.001$; burden score: $\rho = 0.391$, $p < 0.001$). The caregivers' latent desire to die in order to escape from the burden was observed consistently in 0.9% of caregivers, often in 2.6% of them, sometimes in 5.3%, occasionally in 17.5%, and 73.7% of them never had the desire. The desire was also related with both the ZBI and the burden score (ZBI: $\rho = 0.442$, $p < 0.001$; burden score: $\rho = 0.396$, $p < 0.001$).

Furthermore, the caregivers' satisfaction score was highly associated with the ZBI and the burden score (ZBI: $r = -0.490$, $p < 0.001$; burden score: $r = -0.343$, $p < 0.001$). The overall satisfaction was related with the presence of an intimate counselor ($p = 0.006$), but

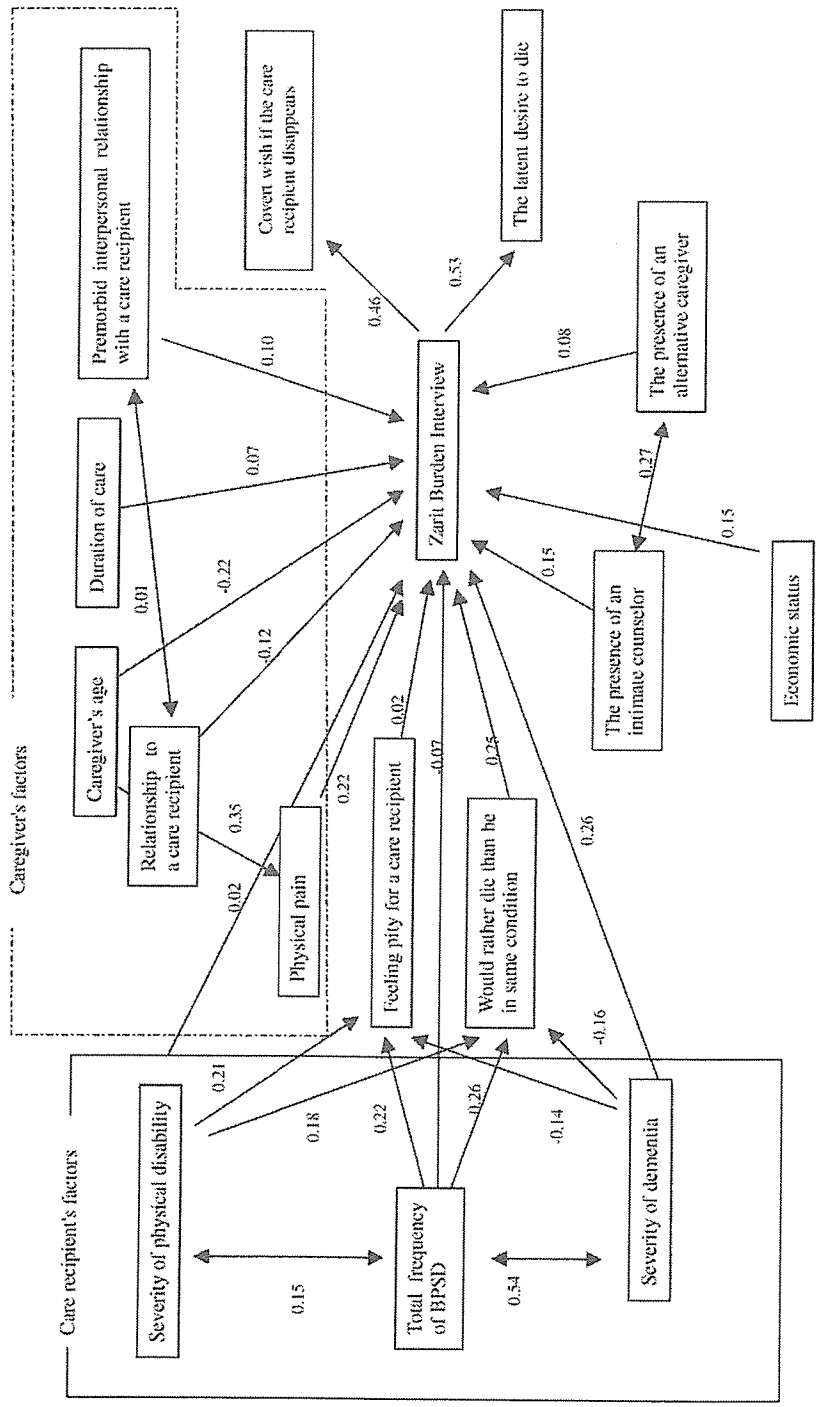

Fig. 2. The path model of the ZBI and the variables. The numbers show the standardized direct effects.

no significant association was found with the age of the caregivers, the relationship, the duration of care, annual income, the presence of an alternative caregiver, the presence of physical pain or the premorbid interpersonal relationship between the caregiver and the care recipient.

3.6. Path analysis (Fig. 2)

A path analysis indicated a significant network model of the relationship around the caregiver's burden, showing an adjusted R^2 of 0.370. The severity of dementia, the sense of "would rather die than be in the same condition" and physical pain of the caregivers showed large direct influences on the score of ZBI. The total frequency of BPSD significantly affected both pity for the care recipients and the sense of "would rather die than be in the same condition", but the burden was significantly influenced only by the sense of "would rather die than be in the same condition".

4. Discussion

In the present study, the ZBI was used to assess the caregivers' burden. The reliability and the validity of ZBI for the Japanese population have already been examined by Arai et al. (1997). However, despite the fact that the ZBI was originally developed for the caregivers of care recipients with dementia, none of the reports had adequately addressed the utility of the interview in assessing caregiver's burden in dementia care.

Caring for a patient suffering from dementia tends to keep caregivers bound at home, thereby increasing the caregiver's burden. The difficulty of coping with such unpredictable patient behavior as sudden tantrums or wandering often distresses the caregiver (Haley, 1997). The strong association of urinary and fecal incontinence with caregivers' burden observed in this study is consistent with previous reports demonstrating that incontinence is a strong predictor for collapse in caregiving at home (Ouslander et al., 1990; etc.). Moreover, the results demonstrated that patients' urinary and fecal incontinence make it difficult for caregivers to continue their regular job or to take time away from caregiving due to the time required for this special care. Hence, it may be essential to pay particular attention to the management of a patient's continence in terms of reducing caregivers' burden. In addition to incontinence, we confirmed strong associations of various BPSD, such as nocturnal delirium, hallucination, interfering with family conversation, and appears unhappy or depressed, with the caregiver's sense of burden.

Pearlin et al. (1990) constructed a model in which the primary stressor can be added to and modified by the care environment and the caregivers' background as secondary stressors. The present study showed that the duration of care, the presence of an intimate counselor, and the presence of physical pain in caregivers were strongly associated with the burden.

In the present study, regression analyses could not construct models with a high, adjusted R^2 for the ZBI and the burden score. But the path analysis indicated a network model with a higher adjusted R^2, which yielded abundant information of the variables involved with care burden. The path analysis showed strong direct effects of severity of

dementia, the sense of "would rather die than be in the same condition", and physical pain on the caregiver. The fact that pity for the care recipient has only little direct effect for the ZBI might suggest that the caregiver's sense of burden stems from concerns of the caregiver's own rather than from his/her compassion for the care recipient. We believe that the network model clarifies the relative involvement and kinetics of various factors influencing the caregiver's burden. In particular, the presence of an intimate counselor to the caregivers and physical pain had substantial impact on caregiver's burden. The overall frequency of BPSD had a significant effect on the caregivers' sense of pity or the desire that they would rather die than be in the same condition. Meanwhile only the feeling that they would rather die than be in the same condition had the largest effect on the caregiver's burden. These results may imply that it is the depreciation of demented care recipients as human, not the sympathy for them that loads the caregivers' mind with the burden.

The caregiver's burden is a complicated concept because it has a multi-layered structure. The structure consists of various factors such as the care recipients' illness, and physical, psychological, and social stress for the caregiver.

In conclusion, the path analysis in the present study revealed the kinetic and dynamic interactions of factors affecting caregiver's burden. The results indicate that caregiver's burden can be affected by physical and psychological status of caregivers, as well as by medical conditions of care recipients. The application of this analytical method may help to establish strategies to reduce caregivers' burden by a better understanding of how the burden develops.

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ORIGINAL PAPER

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Depletion of cholinergic neurons in the nucleus of the medial septum and the vertical limb of the diagonal band in dementia with Lewy bodies

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Abstract The cholinergic basal forebrain is divided into four subregions (Ch1–4), and cholinergic neuronal loss in the nucleus basalis of Meynert (Ch4) has been correlated with cognitive impairments in both Alzheimer's disease (AD) and dementia with Lewy bodies (DLB). However, the Ch1–2 regions, which provide the major cholinergic innervation to the hippocampus, have not been investigated in DLB. The purpose of this study was to reveal the cholinergic neuronal changes in the medial septum (Ch1) and the nucleus of the vertical limb of the diagonal band (Ch2) of DLB brains. Using choline acetyltransferase (ChAT) immunohistochemistry, we showed that the number of ChAT-immunoreactive neurons in DLB brains was significantly lower than the numbers in AD and non-demented (control) brains. No significant difference in the number of ChAT-immunoreactive neurons was found between the AD and control brains. Moreover, the size of the ChAT-immunoreactive neurons was significantly smaller in the AD and DLB brains than in the control brains. These results show that cholinergic neurons of the Ch1-2 regions are more severely affected in DLB than in AD. Our DLB cases did not fulfill the neuropathologic criteria for definite AD. Furthermore, some Lewy bodies were observed in the Ch1-2 regions. Thus, cholinergic neuronal loss in the Ch1-2 regions might be specific to the pathology of DLB. Taking the distribution of cholinergic fibers in the hippocampus into consideration, this study suggests a possibility that hippocampal cholinergic projection is involved in Lewy-related neurites in the CA2–3 regions, the origin of which remains unclear.

Keywords Septal nuclei · Basal forebrain · Immunohistochemistry · Choline acetyltransferase · Cholinesterase inhibitor

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Introduction

Dementia with Lewy bodies (DLB) is the second most frequent neurodegenerative dementing disorder after Alzheimer's disease (AD) [1, 18]. Hippocampal pathology is important in DLB as well as AD, since memory impairment, a chief symptom of both the disorders, is closely related to degeneration of the hippocampus. Lewy-related neurites are usually observed in the CA2–3 regions of the hippocampus in DLB [6, 7, 9, 11, 18], whereas these regions are relatively preserved in AD. In addition, immunoelectron microscopic examinations have revealed that these Lewy-related neurites are distal axons that have undergone change. These neurites are partially immunostained with a neurofilament antibody, but not with a tyrosine hydroxylase antibody [7]. Although the origin of Lewy-related neurites has been poorly understood, the absence of the degenerating axon terminals in hippocampal regions other than the CA2–3 regions of DLB brains might indicate that this pathology is associated with impairments of the hippocampal projection.

Cholinergic neurons in the basal forebrain form a cholinergic column comprising the medial septum, the nucleus of the diagonal band, and the nucleus basalis of Meynert. Mesulam et al. proposed the nomenclature of Ch1 through 4 to designate the various subdivisions of the basal forebrain cholinergic neurons [19]. The medial septum (Ch1) and the vertical limb of the nucleus of the diagonal band (Ch2) provide the cholinergic innervation of the hippocampal formation, and the nucleus basalis

(Ch4) projects to the amygdaloid nuclei and the cerebral cortex [15, 22]. Choline acetyltransferase (ChAT) activity in the cerebral cortex, a decrease in which is correlated with neuronal loss in the nucleus basalis of Meynert (Ch4), has been reported to be involved in cognitive function [16]. Moreover, the reduction of the neocortical presynaptic cholinergic inputs is more severe in DLB brains than in AD brains. Therefore, cholinesterase inhibitors are thought to be more effective in DLB patients than in AD patients [2, 10, 26]. On the other hand, cholinergic neurons within the hippocampal projecting nuclei of the Ch1–2 regions are minimally affected in AD brains as compared with non-demented brains [21, 23]. These neurons have not yet been investigated in DLB brains.

A previous study of ChAT-like immunoreactivity in hippocampal formation in humans showed that punctuate ChAT-immunostaining was conspicuous in the stratum pyramidale of the CA2–3 regions [27]. In the current study, we hypothesized that the Ch1–2 cholinergic neurons projecting to the hippocampus are involved in hippocampal CA2–3 pathology. To test this hypothesis, we performed a qualitative and semiquantitative ChAT-immunohistochemical analysis of cholinergic neurons over the entire length of the Ch1–2 regions of DLB brains, AD brains and non-demented control brains, and compared the results.

Materials and methods

Subjects

Fifteen autopsied brains from eight DLB cases (DLB group: mean age, 75.0 ± 12.6 years; mean brain weight, 1,200 ± 119 g), four AD cases (AD group: mean age, 83.5 ± 0.5 years; mean brain weight, 1,090 ± 43 g) and three non-demented control cases (control group: mean age, 83.3 ± 3.5 years; mean brain weight, 1,145 ± 123 g) were examined in the present study. DLB was diagnosed based on the neuropathological criteria of DLB and divided into five limbic types and three neocortical types [18]. Four AD cases fulfilled the neuropathological criteria of AD according to the NIA-RI-criteria [31]. The progression of Alzheimer pathology was classified from stages 1 to 6 by Braak staging [4]. Neither the DLB group nor the control group fulfilled the pathological criteria for AD [17, 31]. No significant difference was found in mean age or mean brain weight among the three groups. The mean values of the final mini-mental state examination (MMSE) cognitive score before death were also compared between the DLB group and AD group [8], and no significant difference was found (mean MMSE for the DLB group: 4.5 ± 4.7; for the AD group: 4.0 ± 8.0). Informed written consent was obtained from the patients' guardians before carrying out the dissection, and study design was approved by the ethics committee of the Choju medical institute.

Immunocytochemistry

Tissue blocks including the septal nucleus and the vertical limb of the diagonal band were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), embedded in paraffin and cut into serial coronal 7-μm-thick sections. The region studied extended from the anterior border of the septal nucleus to the anterior commissure. Sections taken every 280 μm, i.e., every 40th section were stained with hematoxylin-eosin (HE). By this method, we could examine the entire medial septal nucleus and vertical limb of the diagonal band. After the limits were decided, serial sections (seven to ten sections for each brain) were pretreated with 0.1% trypsin diluted with 0.1% $CaCl_2$ for 20 min, and then immunostained with polyclonal goat anti-ChAT antibody (1:100; Chemicon, Temecula, CA, USA) and monoclonal mouse anti-phosphorylated α-synuclein antibody (1:1,000; Wako, Tokyo, Japan) overnight at 4°C. Alfa-synuclein-immunostaining was performed to confirm the presence of Lewy bodies in the Ch1–2 regions in cases where bodies were observed by HE staining. These primary antibodies were diluted with phosphate-buffered saline (PBS) containing 3% normal goat serum. After incubation with the primary antibodies, the sections were treated with biotinylated secondary antibodies for 2.5 h at room temperature, followed by incubation in avidin-biotinylated horseradish peroxidase (HRP) complex (ABC Elite Kit; Vector, Burlingame, CA, USA) for 0.5 h at room temperature. Immunolabeling was visualized with 3,3′-diaminobenzidine (DAB; Dojindo, Kumamoto, Japan) and nickel ammonium sulfate. Before α-synuclein immunostaining, sections were pretreated with 98% formic acid for 3 min. To investigate the AD pathology in the Ch1–2 regions, the sections from all the brains were also stained by the Gallyas–Braak method.

Semiquantitative analysis of ChAT-immunoreactive neurons

ChAT-immunoreactive neurons in the Ch1–2 regions were semiquantitatively analyzed to investigate the depletion of cholinergic neurons. The number of ChAT-immunoreactive neurons was counted on each section by the two investigators (H.F. and D.I.) who were blinded to the groups. The assessments were repeated and any discrepancies between the evaluations of the two raters were resolved through discussion. The numbers of ChAT-immunoreactive neurons on each of the serial sections at 280 μm intervals were summed, and this value served as a substitute for the total number of neurons in each case.

Densitometric analysis of ChAT-immunoreactive neurons

ChAT-immunoreactivity was investigated by densitometric analysis to detect the depletion of cholinergic

activity in each neuron. The sections were examined under an Olympus microscopy with Olympus AX80T at a total magnification of 200 times. All of the digital images of ChAT-immunoreactive neurons were converted to grayscale using Photoshop 4.0J image processing software (Adobe Systems, Tokyo, Japan) and the image analysis was performed using Scion Image Beta software, Version 4.02 (Scion, Frederick, MD, USA) to determine the pixel density of ChAT-positive cells at a given threshold level. In the present study, the pixel counts of the ChAT-positive neurons determined by image analysis served as a substitute for the cell surface measurement in each neuron. ChAT-immunoreactive neurons in the Ch2 region were randomly selected and the cell surface measurement was performed (pixels/cell). The average cell surface value was calculated for 100 neurons in each brain, followed by the average cell surface value for each of three groups.

Statistical analysis

The data are expressed as the mean ± SEM. The Kruskal–Wallis test followed by the Mann–Whitney *U*-test was used to compare differences among the three groups. p values below 0.05 were considered statistically significant. All statistical analysis was performed using StatView software (Version 5.0) for Windows.

Results

ChAT-immunoreactive neurons were observed from the medial septum (Ch1) to the nucleus of the vertical limb of the diagonal band (Ch2) in all cases. The Ch2 merges with the caudal portions of the Ch1; the Ch2 is located ventral to the decussation of the anterior commissure and reaches the posterior edge of the ventral striatum.

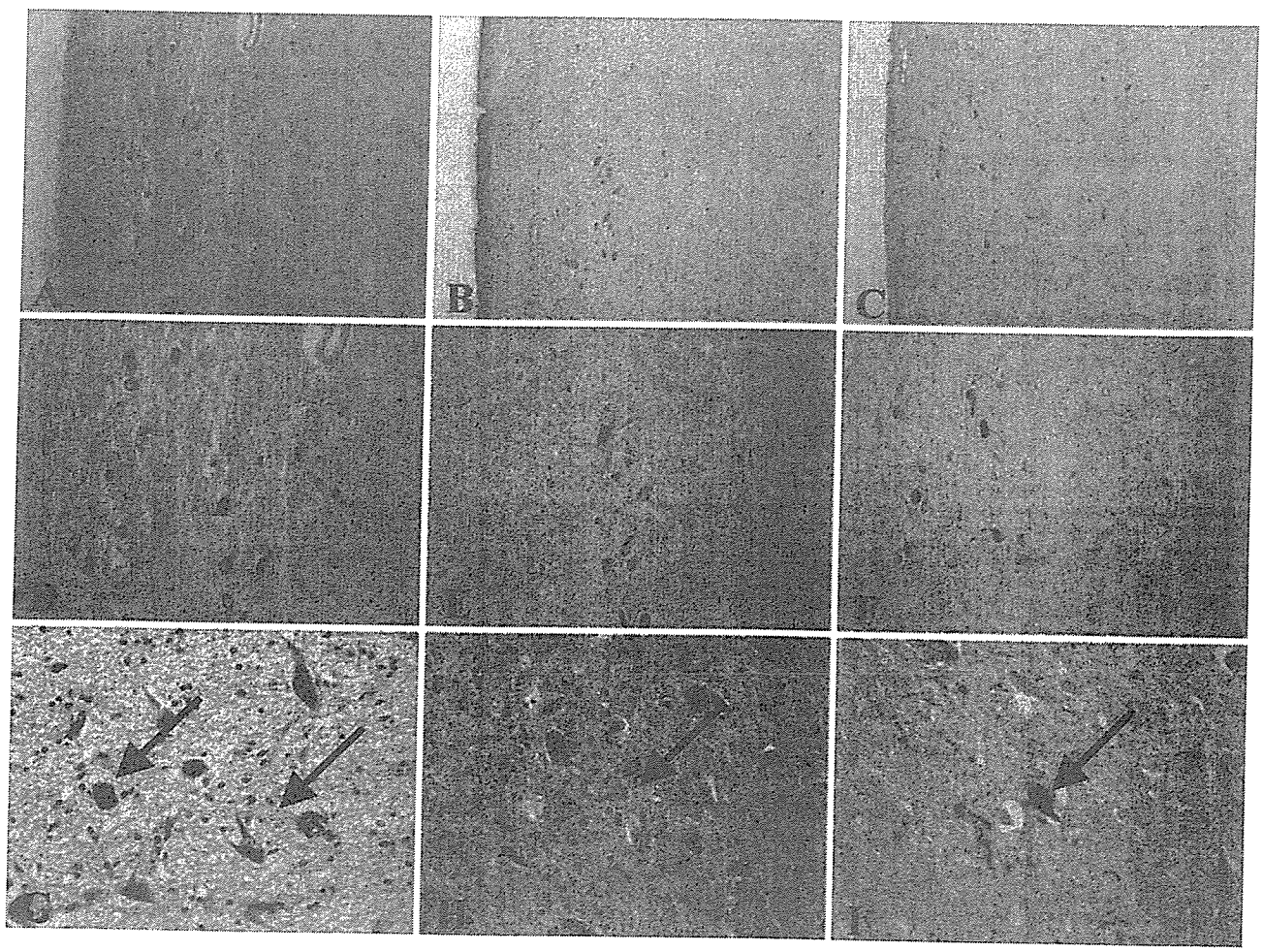

Fig. 1 **a** and **d** ChAT-immunoreactive neurons in the Ch1–2 regions of case 2 (non-demented). **b** and **e** ChAT-immunoreactive neurons in the Ch1–2 regions of case 4 (AD). **c** and **f** ChAT-immunoreactive neurons in the Ch1–2 regions of case 9 (DLB). ChAT immunostainings are shown at a total magnification of 100 times (**a–c**) or 200 times (**d–f**). **g** Neurofibrillary tangle (*NFT*) (*arrows*) in the Ch1–2 regions of the AD brain at a total magnification of 400 times by Gallyas–Braak staining. **h** and **i** Lewy bodies (*arrow*) in the Ch1–2 regions of the DLB brain at a total magnification of 400 times by HE staining and α-synuclein immunostaining

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Cognitive function in Japanese elderly with type 2 diabetes mellitus

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Abstract

The current study was conducted to investigate the cognitive function in Japanese elderly with type 2 diabetes mellitus (DM). Participants included 69 diabetic and 27 nondiabetic subjects (60 to 85 years old). The cognitive functional tests conducted were the Mini-Mental State Examination (MMSE), Word Lists Recall (immediate, delayed), Digit Symbol Test (Wechsler Adult Intelligence Scale—Revised [WAIS-R]), and the Stroop Color Word Test. Hemoglobin A1c (HbA1c) was measured as the index of glycemic control, and information about recent hypoglycemic episodes was gathered by using questionnaires. Student's t test showed that DM subjects had significantly lower scores in the MMSE ($P<.01$) and Digit Symbol Test ($P<.05$) than non-DM subjects. The scores of the Digit Symbol Test in diabetes subjects had a significant negative relationship with HbA1c ($r=-.433$; $P<.001$), and insulin-use had a significant relationship with the scores of the MMSE and Digit Symbol Test. Subjects in the DM group were further divided by insulin use. Comparison of insulin-treated DM subjects, non-insulin-treated DM subjects, and nondiabetic subjects by analysis of variance followed by Bonferroni's post hoc test showed that insulin-treated DM subjects had significantly lower scores in the MMSE and Digit Symbol Tests than both non-insulin-treated DM subjects ($P<.05$) and nondiabetic subjects ($P<.01$). Our study suggests that Japanese elderly DM subjects, especially those with insulin treatment, have poor cognitive function.

Keywords: Digit Symbol Test; MMSE; HbA1c; Insulin; Hypoglycemia

1. Introduction

Cognitive function in elderly diabetes mellitus (DM) subjects has been of interest for more than 80 years and has been explored in several studies; however, the outcomes of these studies have not been entirely conclusive (Strachan, Deary, Ewing, & Frier, 1997). Although most studies have concluded that cognitive performance is worse in elderly DM subjects (Gradman, Laws, Thompson, & Reaven, 1993; Miles & Root, 1922; Perlmuter et al., 1984; Reaven, Thompson, Nahum, & Haskins, 1990), some studies have reported that cognitive function in type 2 DM subjects is comparable to that in non-DM subjects (Atiea, Moses, & Sinclair, 1995; Mattlar, Falck, Ronnemaa, & Hyyppa, 1985). These studies have been performed mainly in Western countries. Because cognitive functional tests are based on language communication, studies should be performed using subjects with different genetic and cultural backgrounds in different languages.

Among the factors involved in the mechanism of cognitive impairment in DM subjects, glycemic control may be one of the most important (Gradman et al., 1993; Meneilly, Cheung, Tessier, Yakura, & Tuokko, 1993). Few studies have investigated the relationship between glycemic control and cognitive function in DM patients. However, one study reported that glycemic control, as measured by hemoglobin A1c (HbA1c) levels, showed a significant negative correlation with cognitive function in DM patients (Reaven et al., 1990). Another reported that oral hypoglycemic medication improved some domains of cognitive ability (attention/concentration, new learning, and problem solving) (Gradman

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et al., 1993). To maintain glycemic control, a combination of several kinds of treatments—including diet regulation, oral medication, and/or insulin treatment—is needed for DM patients. Large prospective studies have shown that persons with DM are at an increased risk of developing dementia, including Alzheimer's disease, particularly when treated with insulin (Ott et al., 1996, 1999). However, the effects of various treatments on cognitive function in DM patients have not been well investigated. For example, there has been only one study—that of Jagusch, Cramon, Renner, & Hepp, 1992—on the effect of treatment in nondemented DM patients; the results showed that insulin-treated subjects had slower simple reaction times. Recently we investigated the effect of treatment on the brain atrophy in elderly DM subjects, and found that the insulin-treated group had the most severe atrophy (Ushida et al., 2001).

Given this situation, the present study was initiated with the following three goals. First, we compared the domains of cognitive functional tests in elderly Japanese subjects (age >60 years) with type 2 DM with a group of elderly non-DM subjects (age >60 years). Second, we wanted to determine whether there was any correlation between the measures of cognitive function and the degree of glycemic control in patients with type 2 DM. Third, we investigated the effect of DM treatment on the performance of cognitive function tests.

2. Subjects and methods

2.1. Subjects

There were 69 subjects with type 2 DM and 27 non-DM subjects. All subjects were outpatients at Nagoya University Hospital in Aichi, Japan, at Gifu Prefectural Tajimi Hospital in Gifu, Japan, at Chiaki Hospital in Aichi, Japan, or at Aoki Kinen Hospital in Mie, Japan. They ranged in age from 60 to 85 years old. All subjects with diagnosis of dementia, depressive disorders by the clinical criteria defined by DSM-III-R (American Psychiatric Association, 1987) or DSM-IV (American Psychiatric Association, 1994), respectively, or any other diseases known to affect cognitive function, or subjects who had cerebral infarctions of more than 1 cm in diameter as visualized by brain CT or MRI, and/or had neurological sings or symptoms, and/or clinical histories of stroke including transient ischemic attacks were excluded. No subjects had audio–visual deficiencies sufficient to impair their performance in the cognitive functional assessments. All participants were independent in terms of performing their daily activities.

An ethical committee approved the study protocol and all patients gave their written informed consent prior to the investigation. After the provision of informed consent, the cognitive functional tests were administered individually to each subject. HbA1c was measured as a marker of glycemic control. DM patients were asked if they had had any hypoglycemic episodes during the recent month over the last month by questionnaire. At the day of the assessment subjects had breakfast as usual and the assessment was performed before noon. The doctors checked the physical conditions of the subjects before the assessment and confirmed that they were not hypoglycemic. Hypertension was diagnosed as follows: prescription of antihypertensive medicine, systolic blood pressure (SBP) of 160 mm Hg or higher, and/or diastolic blood pressure (DBP) of 95 mm Hg or higher. The diagnosis of hyperlipidemia was based on the *Japan atherosclerosis society guidelines for diagnosis and treatment of atherosclerotic cardiovascular disease* (Japan Atherosclerosis Society, 2002). Regarding complications of

Table 1
Characteristics of participants by diabetes status

Variable	DM subjects	Non-DM subjects	*P* value
N	69	27	–
Age	71.6 ± 5.6	73.4 ± 6.6	0.164
Gender (% female)	70.4	52.2	0.107
Education (years)	10.4 ± 2.7	11.4 ± 3.0	0.167
Hypertension (%)	52.5	50.0	0.845
Hyperlipidemia (%)	36.5	60.0	0.074
HbA1c (%)	8.0 ± 1.0	5.7 ± 0.4	*P*<.01

Data are the mean ± S.D. unless otherwise indicated.
Student's unpaired *t*–test (age, education, HbA1c) and Kruskal–Wallis analysis (other variables).

Table 2
Performance on measures of cognitive function by diabetes status

Measure	DM subjects	Non-DM subjects	*P* value
MMSE	27.1 ± 2.2	28.3 ± 1.7	*P*<.05
Word List (immediate)	5.7 ± 1.7	6.2 ± 1.7	0.254
(delayed)	7.1 ± 2.2	6.7 ± 2.0	0.364
WAIS-R Digit Symbol	36.3 ± 10.9	43.0 ± 12.1	*P*<.05
Stroop Color Word Test	19.2 ± 12.8	15.0 ± 6.7	0.113

Data are the means ± S.D., unless otherwise indicated.
A higher score indicates better performance, except in the case of the Stroop Color Word Test.
Student's unpaired *t*–test.
WAIS-R: Wechsler Adult Intelligence Scale-Revised.

Table 3
Correlation coefficients between scores of cognitive tests and diabetic characteristics

Variables	MMSE	WAIS-R
WAIS-R Digit Symbol	0.456**	–
Diabetes duration	0.078	−0.155
HbA1c	−0.205	−0.433**
Neuropathy	−0.075	0.005
Nephropathy	−0.008	−0.021
Retinopathy	−0.095	0.015
Hypoglycemia	−0.265	−0.229
Insulin-treatment	−0.379**	−0.304*

Pearson's correlation coefficients analysis (MMSE, WAIS-R digit symbol, Diabetes duration HbA1c) and Spearman's correlation coefficients analysis (other variables).
* *P*<.05.
** *P*<.01.