Neocortical Synapse Density and Braak Stage in the Lewy Body Variant of Alzheimer Disease: A Comparison with Classic Alzheimer Disease and Normal Aging

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Abstract. Substantial numbers of cortical and subcortical Lewy bodies are seen in approximately one quarter of patients whose brains show sufficient histopathologic changes for a neuropathologic diagnosis of definite Alzheimer disease (AD). This subset of cases has been named the Lewy body variant of AD (LBV). Despite comparable dementia and the presence of neocortical senile plaques in LBV patients, the overall burden of neuropathologic changes, in particular neurofibrillary tangles (NFT), is less than in classic AD. While NFT frequency correlates with dementia severity in classic AD, the cognitive impairment in patients with LBV cannot be completely explained by such changes. Since several studies have suggested a role for synapse loss in relation to dementia severity in classic AD, we decided to investigate the role of synapse loss as a candidate for the cognitive impairment of LBV.

The Braak staging method is based upon the distribution and severity of neurofibrillary changes, and one therefore would expect LBV cases to be assigned to lower Braak stages. In the present study we assigned a Braak stage to 14 LBV cases, 31 classic AD cases, and a group of 10 non-demented aged controls. We compared the severity of synapse loss as determined by ELISA immunoassay for synaptophysin and Braak stage among the three diagnostic groups. When compared to normal controls, synaptophysin concentrations were statistically significantly lower in both demented groups. There was comparable synapse loss in LBV and AD despite significantly lower Braak stages in the LBV cases. These results suggest a major role for loss of synapses as the substrate of cognitive impairment in LBV.

Key Words: Alzheimer disease; Braak staging; Lewy body disease; Synaptophysin.

INTRODUCTION

Approximately one quarter of patients with sufficient histopathologic changes for the diagnosis of definite Alzheimer disease (AD) also have substantial numbers of cortical and subcortical Lewy bodies (LB) (1, 2), as seen in idiopathic Parkinson disease (PD) (3–6). Clinically, in addition to memory retrieval difficulties, these patients often have attentional deficits, visuospatial deficits, fluctuation in cognitive function, visual hallucinations, neuroleptic sensitivity, and occasionally evidence of parkinsonism, specifically rigidity and bradykinesia (7–10). This subset of cases has been referred to as the Lewy body variant of AD (LBV) (11, 12). By definition, patients with LBV meet established neuropathologic criteria for the diagnosis of AD (11, 12). However, despite comparable, or even more frequently, greater cognitive impairment in these patients (13, 14), the overall burden of AD-type neuropathologic changes, in particular neurofibrillary tangles (NFT), is less than in classic AD (9, 11, 12).

In classic AD, it has been shown that while cortical senile plaque density does not correlate well with the severity of clinical dementia (15, 16), neocortical NFT frequency, on the other hand, does (15–17). A recently popularized method for the staging of AD related changes, focusing on neurofibrillary pathology, has been developed by H. and E. Braak (18). A handful of studies has demonstrated a correlation between clinical severity of AD and Braak stage (19–21), with patients having lower Braak stages tending to show a lesser degree of cognitive impairment. Since the Braak staging method is based upon the distribution and severity of neurofibrillary changes, one would expect LBV cases to be assigned to lower Braak stages than AD patients at similar clinical stages (22). If that were confirmed, the cognitive impairment in patients with LBV would not be fully explained by neocortical neurofibrillary pathology.

Several studies have suggested a role for neocortical synapse loss in relation to dementia severity in classic AD (23–25). A recent investigation found comparable hippocampal synapse density loss in LBV and AD (26), and a separate report demonstrated similar synapse density in the 2 groups in the midfrontal gyrus (27). The present study evaluates global synapse density in LBV cases compared to classic AD cases, hypothesizing that synapse density correlates better with level of cognitive function than Braak stage in LBV. We retrospectively examined 14 LBV cases, 31 “typical” AD cases, and a group of 10 non-demented aged controls. We assigned each a Braak stage and compared the groups relative to severity of synapse loss and Braak stage.
MATERIALS AND METHODS
Case Selection and Diagnostic Evaluation

We retrieved 14 autopsy cases with a neuropathologic diagnosis of LBV (9 male, 5 female; age of death 75.2 years ± (7.1) (X ± (SD)); duration of disease 7.2 years ± (3.3); postmortem interval 12.1 hours ± (6.2)). At least 16 histologic sections were reviewed from each case for evidence of neuropathologic changes of AD, PD, LBV, or other neuropathologic disorder using hematoxylin and eosin (H & E), thioflavine S, and ubiquitin stained paraffin sections. We also retrieved 31 randomly selected autopsy cases that had changes sufficient for a neuropathologic diagnosis of definite AD, and in which no LB was identified in H&E stained brainstem sections (12 male, 19 female; age of death 79.6 years ± (6.1); duration of disease 8.5 years ± (3.2); postmortem interval 12.8 hours ± (4.7)). All 45 cases were from patients with documented cognitive impairment who had been evaluated in the Alzheimer Disease Center (ADC) at the University of Texas Southwestern Medical Center at Dallas. Cognitive testing included the Mini-Mental Status Exam (MMSE) (28), the Blessed Information-Memory-Concentration Test (IMC) (29), and the Clinical Dementia Rating Scale (CDR) (30). At least 1 of these testing methods was utilized on all of the LBV cases and 27 of 31 AD cases. The mean interval between last examination and death was comparable in the 2 groups: 2.5 years ± (2.5) for the AD group and 2.0 years ± (3.0) for the LBV group. The AD and LBV groups demonstrated similar levels of cognitive impairment [MMSE: AD = 10.8 ± (5.7), LBV = 12.4 ± (7.5), (p = 0.51), t-test]; Blessed: AD = 12.1 ± (5.0), LBV = 8.9 ± (4.1), (p = 0.17); CDR: AD = 3.3 ± (1.6), LBV = 2.5 ± (0.9), (p = 0.078)].

Control cases consisted of 10 aged non-demented individuals with NP insufficient for a diagnosis of AD (9 male, 1 female; age of death 73.0 years ± (9.7); postmortem interval 11.7 hours ± (8.0)). These included 6 cases without neocortical NP and 4 cases with mild to moderate (CERAD scores 1 or 3) neocortical NP. Although formal cognitive testing was not performed on this group of cases, 4 of the 10 controls were seen by either a neurologist or a psychiatrist, most commonly for depression following the death of a spouse. The remaining 6 controls all had chronic medical conditions and were seen routinely by their physicians up to the time of death.

Autopsy diagnostic criteria for AD were based upon the identification of SP in appropriate density in the hippocampus and neocortex according to CERAD criteria (31). Cases with other lesions (e.g. Pick bodies, infarcts) that might indicate a concomitant or alternative diagnosis were excluded from this study.

Ubiquitin Immunohistochemistry

Ubiquitin immunostaining was performed in standard fashion at room temperature on a BioTek Solutions TechMate® 1000 automated immunostainer (Ventana BioTek Systems, Tucson, Ariz.). Briefly, paraffin sections of substantia nigra and superior temporal gyrus, insula, or cingulate gyrus were cut at 3 µm on a rotary microtome, mounted on positively charged glass slides (POP100 capillary gap slides, Ventana BioTek Systems), air dried overnight, deparaffinized, and placed in 200 ml heat-in-

\[\text{Table 1: Adaptation of Braak Staging Using CERAD Neuropathology Protocol}\]

<table>
<thead>
<tr>
<th>Braak stage</th>
<th>Entorhinal NFT score</th>
<th>Hippocampus NFT score</th>
<th>Sum of neocortical NFT scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>1, 3</td>
<td>1</td>
<td>0</td>
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<tr>
<td>III</td>
<td>3, 5</td>
<td>3, 5</td>
<td>1–3</td>
</tr>
<tr>
<td>IV</td>
<td>3, 5</td>
<td>3, 5</td>
<td>4–14</td>
</tr>
<tr>
<td>V</td>
<td>3, 5</td>
<td>3, 5</td>
<td>4–14</td>
</tr>
<tr>
<td>VI</td>
<td>5</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

(0 = Absent, 1 = Sparse, 3 = Moderate, 5 = Frequent) * From S. S. Mirra, personal communication

Note: The Neocortical NFT Score represents the sum of middle frontal, superior temporal, and inferior parietal CERAD NFT scores. For example, a case with severe NFTs in the entorhinal region (CERAD score of 5), severe NFTs in the hippocampus (CERAD score of 5), and moderate NFTs in all neocortical regions (CERAD scores of 3 + 3 + 3 = 9) would be assigned a Braak stage of V using this technique.

A modification of the originally described Braak staging system of AD-related changes was employed in the present study, adapted from the CERAD Neuropathology Protocol (S.S. Mirra, personal communication). Thioflavine S stained sections of hippocampus at the coronal level of the mamillary bodies, the hippocampus at the coronal level of the lateral geniculate body, the middle frontal gyrus, the inferior parietal lobule, and the superior temporal gyrus were evaluated in all cases by fluorescent microscopy for neurofibrillary tangles. The density of tangles in each section was graded using CERAD criteria (i.e.: 0 = Absent, 1 = Mild or sparse, 3 = Moderate, 5 = Severe or frequent). The 6 values obtained from the 3 sets of paired bilateral cortical sections were summed and then halved to generate a total neocortical value. Cases were then assigned a Braak stage as shown in Table 1.
Quantification of Neocortical Synaptophysin

At the time of autopsy, neocortical gray matter was dissected from the middle frontal, superior temporal, and inferior parietal regions bilaterally, snap-frozen in LN<sub>2</sub>, and stored at −70°C. Frozen tissue samples were subsequently thawed, weighed, homogenized in Tris-buffered saline (10 mM, pH 8.0, 5% non-fat dry milk, and 0.2% Tween-20). We used a 2-site, 2-antibody "sandwich" ELISA technique, first binding monoclonal antibody to synaptophysin (Chemicon, Temecula, Calif.) (1:2,000 dilution, incubated overnight at 4°C) directly to the plate, followed by blocking buffer, the test samples (incubated overnight at 4°C), a rabbit polyclonal antibody to synaptophysin (Zymed, South San Francisco, Calif.) (1:200 dilution) and then alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, Calif.) (1:1,000 dilution) with para-nitrophenyl phosphate as the chromogen. Plates were read on a Bio-Rad microtiter plate reader using a 405 nm filter. Each test sample was analyzed in triplicate.

In order to make the assay more quantitative and to allow normalization of data between runs, a standard curve was constructed for each plate using 9 serial 2-fold dilutions (1:100 through 1:25,600) of autopsy neocortex from a young, neurologically normal control with a short postmortem interval. A value of 1 arbitrary concentration unit (acu) was assigned to the well containing the highest dilution of the reference standard. The same series of dilutions of the same reference case was used to generate the standard curve for each plate assayed. We used a 4-parameter logit-log regression curve fit routine (absorbance vs log[concentration]) to determine the range of optical densities over which there was a linear relationship between antigen concentration and optical density. This curve fit routine is especially useful for analyzing data that generate sigmoid curves. Synaptophysin concentration in each test sample (relative to the reference case) was determined from the standard curve. Only absorbance data that fell within the linear portion of the standard curve were used. An assay was repeated if the coefficient of variation between the triplicate samples exceeded 15%.

Analysis

For each case included in the present study, a mean neocortical synaptophysin concentration was generated from bilateral frontal, temporal, and parietal regions. A neocortical mean was utilized for comparison to account for regional variability and occasional asymmetry in synaptophysin concentration from case to case. This allowed for a comparison of global neocortical synapse loss. In addition, analysis of synaptophysin among the 3 groups revealed similar comparisons when each individual lobe (frontal, temporal, and parietal) was evaluated separately. Synapse density and Braak stage were compared among the LBV, classic AD, and control groups. Braak stage comparisons were performed using the exact linear trend test due to the ordinal nature of the Braak staging method. Mean synaptophysin concentration comparisons were performed using the Wilcoxon test, which allowed for comparisons across the 3 groups. A Spearman's correlation analysis was used to compare Braak stage and synaptophysin concentration in the LBV group in a rank order fashion.

RESULTS

As expected, LBV cases were assigned to lower Braak stages overall, when compared to classic AD cases (p < 0.001). The mean Braak stages in the classic AD group, the LBV group, and the control group were 5.4, 3.6, and 1.6 respectively (Figure 1). While there was over-representation of the neocortical stages in the classic AD group, there was a downward shift in the LBV group as recently also noted by Gearing and Mirra (32) with clustering about stages II and V. As expected, there was an over-representation of the lower Braak stages in the normal control group (NC) (Figure 2). There was a moderate negative correlation between synaptophysin concentration and Braak stage in the LBV group, although this

Mean Synapse Density

![Graph showing mean synapse density based on synaptophysin concentration in the Alzheimer disease (AD), Lewy body variant of Alzheimer disease (LBV), and normal control (NC) groups. Synaptophysin values are based on the neocortical frontal-temporal-parietal mean and are given in arbitrary concentration units (acu).](image)

**DISCUSSION**

Although there is some debate as to whether LBV exists as a distinct neuropathologic entity (33), there are histopathologic differences between LBV and classic AD (11, 12). In addition to the presence of neocortical and subcortical LB, the severity of NFT formation in LBV is typically less than in classic AD (9, 11, 12). This was confirmed in our study by a significantly lower mean Braak stage in the LBV group. We were able to show, however, that synapse density, utilizing mean synaptophysin concentration (25), was comparable in the similarly cognitively impaired AD and LBV groups. Based on these findings, we conclude that neurofibrillary pathology cannot fully account for the cognitive impairment seen in LBV, but rather that the loss of synapses, as in classic AD (23–25), is a better candidate for the neuroanatomic substrate of cognitive impairment in these patients. Samuel et al. showed similar results when looking specifically at the midfrontal gyrus using dot blot analysis and immunohistochemistry for synaptophysin (27).

Further supporting this concept is the fact that there was no statistically significant correlation between synaptophysin concentration and Braak stages in our LBV group. This suggests that the presence of neocortical tangles in this group does not significantly contribute to additional loss of neocortical synapses, and that the loss of projections from limbic structures may result in decreased neocortical synapse density.

It is also possible that some of the neocortical synapse loss in LBV results from loss of input from other subcortical structures. In LBV there is involvement of several transmitter-specific subcortical structures that project to the cortex (9, 11), including the dopaminergic substantia nigra; the noradrenergic locus coeruleus, and the cholinergic nucleus basalis of Meynert (nBM). In addition to contributing to the parkinsonism seen in some of these patients, such subcortical pathology may also contribute to cognitive impairment. For example, we know that in

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**TABLE 2**

Regional Synaptophysin Concentration*

<table>
<thead>
<tr>
<th>Cortical region</th>
<th>Normal control group (n = 10)</th>
<th>Alzheimer disease group (n = 31)</th>
<th>Lewy body variant group (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X ± (SD)</td>
<td>X ± (SD)</td>
<td>X ± (SD)</td>
</tr>
<tr>
<td>Frontal lobe</td>
<td>12,186 ± (7,518)</td>
<td>6,847 ± (4,139)</td>
<td>7,273 ± (2,751)</td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>17,093 ± (7,503)</td>
<td>8,702 ± (4,969)</td>
<td>9,637 ± (4,616)</td>
</tr>
<tr>
<td>Parietal lobe</td>
<td>10,143 ± (5,686)</td>
<td>4,919 ± (2,935)</td>
<td>6,105 ± (3,259)</td>
</tr>
</tbody>
</table>

* Mean values in arbitrary concentration units.

Note: Synaptophysin concentration is significantly different among the three groups for each neocortical region (ANOVA, p < 0.01 for each lobe). Bonferroni multiple comparisons (p < 0.05) were utilized for pairwise comparisons of the groups for each cortical region. The mean synaptophysin concentration was significantly less for each neocortical region in the AD group compared to the respective neocortical region in the NC group. The mean synaptophysin concentration was also significantly less for each neocortical region in the LBV group compared to the respective neocortical region in the NC group. The mean synaptophysin concentrations were not statistically significantly different between the AD and LBV groups in any of the neocortical regions.
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general, the deficit in cholinergic input to the neocortex is more extensive in LBV patients than in AD patients (34–36), due largely to greater neuronal damage in the nBM (37). The relatively greater loss of subcortical input to the cortex in LBV might account for the quantitative and qualitative differences in cognitive impairment between AD and LBV, such as fluctuating cognitive impairment, transient clouding, or loss of consciousness, and rapid clinical progression (8–10, 38–40).

A recent study showed a positive correlation between LB counts and dementia severity in LBV (41). Although we know little about the possible pathogenetic relationships between LB formation and synapse loss, it is quite possible that neocortical LB pathology may exacerbate the effects of underlying AD-related synapse loss. One might speculate that LB formation disrupts the neuronal cytoskeleton, resulting in an inability of the neuron to maintain synaptic integrity. On the other hand, it is also possible that LB are merely a secondary phenomenon, generated only after the initiation of synaptic degeneration. In either case, it is interesting to note that neocortical LB are immunoactive for synaptophysin (42). Future investigations might assess the relationship between neocortical LB burden, namely LB counts, with synaptophysin concentration in specific neocortical regions.

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