Old age amyotrophic lateral sclerosis and limbic TDP-43 pathology

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Abstract
This study aimed to assess and compare the burden of transactive response DNA-binding protein of 43 kDa (TDP-43) pathology and clinical features of amyotrophic lateral sclerosis (ALS) in three age groups. All cases were from the Mayo Clinic brain bank for neurodegenerative disorders and most were followed longitudinally in the ALS Clinic. Cases with moderate-to-severe Alzheimer’s disease neuropathological change were excluded. The 55 cases included in the study were divided into three groups by age at death: 75 years or older (old-ALS, n = 8), 64–74 years (middle-ALS, n = 23), and 63 years or younger (young-ALS, n = 24). Clinical features, including disease duration, initial symptoms, and ALS Cognitive Behavior Score (ALS-CBS), were summarized. Sections of paraffin-embedded tissue from the motor cortex, basal forebrain, medial temporal lobe, and middle frontal gyrus were processed for phospho-TDP-43 immunohistochemistry. The burden of TDP-43 pathology was analyzed using digital image analysis. The TDP-43 burden in the limbic system (i.e., amygdala, dentate gyrus and CA1 sector of the hippocampus, subiculum, and entorhinal cortex) was greater in old-ALS than in young-ALS and middle-ALS. TDP-43 burden in the middle frontal gyrus was sparse and did not differ between the three groups. The average of ALS-CBS was not different between the three groups. The present study shows that the amygdala and hippocampus are vulnerable to TDP-43 pathology in older patients with ALS. We discuss the evidence for and against this pathology being related to concurrent limbic-predominant, age-related TDP-43 encephalopathy neuropathologic change.

KEYWORDS
aging, ALS, LATE, limbic system, neuropathology, TDP-43

1 INTRODUCTION
Transactive response DNA-binding protein of 43 kDa (TDP-43) forms neuronal and glial inclusions in a number of neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), a subset of frontotemporal lobar degeneration (FTLD), and limbic-predominant, age-related TDP-43 encephalopathy (LATE) [1–4]. ALS is a fatal neurodegenerative disease that predominantly affects upper and lower motor neurons, resulting in progressive muscle weakness and atrophy, with fasciculations, hyperreflexia, and bulbar symptoms. In addition, approximately 50% of ALS patients develop cognitive impairment, with about 5% developing dementia, referred to as ALS with dementia (ALS-D) [5]. Moreover, some patients with FTLD and TDP-43 pathology (FTLD-TDP) also develop motor neuron disease (FTLD-MND). Therefore, ALS, ALS-D, and FTLD-MND are considered a clinico-pathological spectrum.

In addition to neuronal loss and gliosis, most ALS patients have TDP-43-immunoreactive neuronal cytoplasmic inclusions (NCI) and glial cytoplasmic inclusions...
(GCI) in the pyramidal motor system. Some patients have extramotor TDP-43 pathology. Nishihira et al. classified ALS cases into two types—Type 1 had TDP-43 pathology predominantly in the pyramidal motor system and Type 2 had TDP-43 pathology extending to extramotor areas of frontal cortices, hippocampus, striatum, and substantia nigra [6]. Subsequently, Brettschneider et al. proposed staging of ALS based upon the distribution of TDP-43 pathology, with early stages limited to the pyramidal motor system and later stages displaying TDP-43 pathology in extramotor systems, such as basal ganglia and temporal cortex [7]. The two schemes differ most significantly in whether limbic structures are included or not.

Aging is a risk factor for a range of neurodegenerative disorders and concurrent neuropathologic processes are common with increasing age [8]. In a pathologic study of a large series of the elderly brains, at least one-third had multiple proteinopathies [9]. The presence of multiple pathologies contributes to cognitive decline [10, 11]. Even for disorders typically associated with an early age of onset, comorbid pathologies need to be considered in older patients with these disorders. TDP-43 pathology is a common finding in a number of neurodegenerative disorders, such as Alzheimer’s disease (AD), hippocampal sclerosis, and corticobasal degeneration, as well as some neurologically normal elderly individuals [12–14]. LATE is a recently proposed term for TDP-43 proteinopathy predominantly in the medial temporal lobe in the elderly [4]. A subset of individuals with LATE neuropathologic change also have neuronal loss and gliosis in the hippocampus consistent with hippocampal sclerosis [15]. It remains controversial if LATE can be differentiated from primary TDP-43 proteinopathies in the elderly, such as late-onset FTLD-TDP [16]. The clinical and pathological features of ALS differ considerably from those seen in LATE; however, it is possible that older patients with ALS may have concurrent LATE, but this has not been systematically investigated. The focus of the current study addresses this by examining the frequency and distribution of TDP-43 pathology, especially extramotor pathology, in late-onset ALS.

For this study, we defined three groups—old-ALS, middle-ALS, and young-ALS—with the old-ALS being defined as ALS patients who died at 75 years of age or older, with the middle-ALS being patients who died between 64 and 74, and the young-ALS being patients who died at 63 years of age or younger. We compared the distribution and density of TDP-43 pathology in motor and extramotor regions, and we compared clinical features between old-ALS, middle-ALS, and young-ALS.

2 | METHODS

2.1 | Case selection

All patients included in this study had both a clinical and pathologic diagnosis of ALS and were processed in the Mayo Clinic brain bank for neurodegenerative disorders from 2010 to 2021. All but one patient had been evaluated at Mayo Clinic in Florida or Minnesota, and all but two patients were followed longitudinally in the Mayo Clinic Florida ALS Clinic [17, 18]. Brain autopsies were performed after consent of the legal next-of-kin or someone authorized to provide consent. Deidentified autopsy samples are considered exempt from human subject research according to the 2018 Revised Common Rule (45 CFR 46). The Mayo Clinic brain bank operates under protocols approved by the Mayo Clinic Institutional Review Board.

All cases had systematic and standardized neuropathologic evaluations, with collection of data on concurrent pathologies, such as Alzheimer type neuropathologic change (ADNC) [19]. To be included, all cases had to have tissue from regions of interest in order to conduct digital quantitative burden analysis. A total of 94 brains with pathologic diagnoses of ALS were identified. Cases were excluded if they had pathologic features of chronic traumatic encephalopathy (n = 2), ALS due to hexanucleotide repeat expansion in C9ORF72 (n = 25), or ALS without TDP-43 pathology (ALS-FUS and ALS-SOD1 [n = 3]). One patient carried a mutation in TARDBP (c.892G>A; p.Gly298Ser). Cases were excluded if they had moderate-to-severe ADNC, specifically, Braak neurofibrillary tangle (NFT) stage IV or greater and Thal amyloid phase 4 or 5 (n = 9). The final cohort was 55 patients, all of whom were Caucasian. We defined three groups based on the age at death: old-ALS (≥75) (N = 8), middle-ALS (64–74) (N = 23), and young-ALS (≤63) (N = 24).

2.2 | Neuropathologic assessment

Most of the brains were received with the left hemibrain fixed in 10% formalin; the right hemibrain frozen at −80°C. Formalin-fixed brains underwent systematic and standardized sampling with neuropathologic evaluation by an experienced neuropathologist (D.W.D.). Paraffin-embedded 5-μm-thick sections mounted on glass slides were stained with hematoxylin and eosin (H&E) and with thioflavin S. Braak NFT stage and Thal amyloid phase were assigned based upon lesion density and distribution of senile plaques and NFTs with thioflavin S fluorescent microscopy according to published criteria [20–22]. Immunohistochemistry for phospho-TDP43 (pTDP-43) (pS409/410, mouse monoclonal, 1:5000, Cosmo Bio) was performed on sections of the motor cortex, middle frontal cortex, hippocampus, basal forebrain, midbrain, medulla, and spinal cord to establish a neuropathological diagnosis of ALS. In addition, degeneration of upper and lower motor neurons, as well as corticospinal tract degeneration, were assessed with a myelin stain (Luxol fast blue—periodic-acid Schiff) and immunohistochemistry for microglia (IBA-1, rabbit IG, 1:3000; Wako Chemicals [23]). Bunina bodies were detected on H&E stained sections.
TDP-43 immunohistochemistry was performed on paraffin embedded tissue sections mounted on glass slides of the spinal cord, medulla, basal ganglia, hippocampus, middle frontal cortex and motor cortex with phospho-TDP-43 [7]. ALS cases were classified according to the scheme proposed by Nishihira [6] as either Type 1 (TDP-43 pathology in predominantly the pyramidal motor system) or Type 2 (TDP-43 pathology extending to extramotor areas of frontal and temporal cortices, hippocampus, striatum, and substantia nigra). In addition to formal diagnostic evaluations, slides were reviewed by two observers (A.M. and S.K.) to assess the morphology and severity of TDP-43 lesions in the dentate gyrus, CA1 sector and subiculum of the hippocampus, entorhinal cortex, the white mater of the parahippocampal gyrus, and corticomedial region of the amygdala. The morphology of TDP-43 pathology was categorized as follows: round NCI, preinclusion, NFT-like NCI, ring-shaped NCI, short dystrophic neurites (DN), long and thick DN, dots, GCI, and processes in subpial region [24]. The severity of TDP-43 pathology was graded semi-quantitatively on a 5-point scale (0 = absent, 0.5 = rare, 1 = mild, 2 = moderate, 3 = severe) as previously reported [13].

ADNC was assessed with consensus criteria for the neuropathologic diagnosis of AD [25]. Lewy-related pathology was assessed with α-synuclein immunohistochemistry (NACP, rabbit polyclonal, 1:3000) [26] in the amygdala, basal forebrain, brainstem, cingulate gyrus, and temporal lobe, and classified as the brainstem, limbic and diffuse types [27]. Hippocampal sclerosis (HpScl) was assessed on H&E stained sections of the hippocampus as previously described [28]. Argyrophilic grain disease (AGD) was assessed with phospho-tau immunohistochemistry (CP13, mouse monoclonal, 1:1000 from the late Dr Peter Davies, Feinstein Institute, North Shore Hospital, NY or AT8, mouse monoclonal, 1:2500; DAKO) in the amygdala, and if necessary, the hippocampus and cingulate gyrus. We diagnosed AGD if there are argyrophilic grains with pretangles, balloon neurons, tau-positive
granular fuzzy astrocytes, and oligodendroglial coiled bodies, and AGD was classified into one of three stages as proposed by Saito et al. [29].

2.3 | Quantitative digital image analysis

To quantify pTDP-43 burden, digital image analysis was performed in select brain regions. pTDP43-stained sections of amygdala, posterior hippocampus, middle frontal gyrus, and motor cortex were scanned on ScanScope XT (LEICA Biosystems). The dentate gyrus, CA1 sector, subiculum, entorhinal cortex and white matter of the parahippocampal gyrus, corticomedial region of the amygdala, middle frontal gyrus, and motor cortex were annotated using ImageScope-12.4 and analyzed in Spectrum-12.4 (LEICA Biosystems). The regions of interest were defined based on H&E staining and the atlas. A custom-designed color deconvolution algorithm was used to detect pTDP43-positive pathology (Figure 1). To avoid including nonspecific signals, tissue sectioning artifacts, medium-to-large size blood vessels and perivascular spaces were manually edited out of the region of interest using a negative pen tool [30]. Total pTDP43 burden was expressed as percent ratio of the area of immunoactive pixels to the total pixels in the annotated region.

2.4 | Double-labeling immunofluorescence staining

To assess colocalization of tau and TDP-43, double immunofluorescence staining was performed with CP13 (1:1000) and pTDP-43 (Rb3655, rabbit polyclonal, 1:1000, from Dr. Leonard Petrucelli, Mayo Clinic, Jacksonville) using a method previously described [13]. The deparaffinized and rehydrated sections were blocked with Protein Block plus Serum Free (DAKO) for 1 h and incubated with primary antibodies diluted in with Antibody Diluent with Background-Reducing Components (DAKO) overnight at 4°C. Sections were washed three times with phosphate-buffered saline (PBS) for 5 min each at room temperature and then incubated with secondary antibodies Alexa Fluor 568 (1:500; Thermo Fisher Scientific Inc.) and Alexa Fluor 488 (1:500; Thermo Fisher Scientific Inc.) diluted with Antibody Diluent with Background-Reducing Components for 1.5 h at room temperature in a dark chamber. Sections were washed three times with PBS for 5 min at room temperature, incubated with 1% Sudan Black for 2 min, washed with distilled water, and mounted with Vectashield mounting media containing DAPI (Vector Laboratories). Representative images were taken with a BX50 fluorescent microscope (Olympus Co. Ltd.).

2.5 | Clinical data collection

All but two ALS patients were prospectively followed at Mayo Clinic in Florida. Two neurologists (A.M. and H.S.) abstracted the following information from the electronic medical records that had been recorded throughout the course of disease. Abstracted data were entered into a database and included sex, age at symptom onset, age at death, family history of MND, and ALS Cognitive Behavioral Screen (ALS-CBS) [31] for cognitive function.

2.6 | Statistical methods

All statistical analyses were performed using R version 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria) and EZR (Saitama Medical Center, Jichi Medical University Saitama, Japan), which is a graphical interface for R [32]. A Fisher’s exact test was performed for group comparisons of categorical data, as appropriate. Analysis of one-way ANOVA were used for analyses of continuous variables, as appropriate. p < 0.05 were considered statistically significant. Bonferroni corrections were used to adjust for multiple testing.

3 | RESULTS

3.1 | Demographics and clinical presentations

Demographic and clinical characteristics of the cases are summarized in Table 1. Age at onset was 52 ± 7 years in

| TABLE 1 | Demographics and clinical presentation |
|-------------------|------------------|------------------|------------------|-------------|
|                  | Young-ALS (N = 24) | Middle-ALS (N = 23) | Old-ALS (N = 8) | p Value |
| Male             | 13 (54%)          | 12 (52%)          | 5 (53%)         | 0.94      |
| Age at onset, years | 52 ± 7           | 65 ± 4           | 76 ± 5         | <0.0001   |
| Disease duration, months | 46 ± 28          | 39 ± 25          | 34 ± 29       | 0.47      |
| Age at death, years | 56 ± 6           | 68 ± 3           | 79 ± 4        | <0.0001   |
| Family history of MND | 1 (4%)           | 1 (4%)           | 0 (0%)        | 1         |
| ALS-CBS          | N = 19            | N = 15           | N = 3         |           |
| Final score      | 15 ± 3            | 15 ± 3           | 16 ± 1        | 0.77      |
| Duration final score from death, months | 14 ± 12          | 11 ± 9           | 11 ± 5       | 0.75      |

Abbreviations: ALS-CBS, ALS cognitive behavioral screen; MND, motor neuron disease.
TABLE 2  Pathologic feature

| Pathologic feature                                      | Young-ALS (N = 24) | Middle-ALS (N = 23) | Old-ALS (N = 8) | p Value |
|---------------------------------------------------------|--------------------|---------------------|-----------------|---------|
| Brain weight, grams                                     | 1286 ± 117         | 1253 ± 120          | 1135 ± 199      | 0.03    |
| Nishihira ALS classification                            |                    |                     |                 | 0.003   |
| Type 1 (motor predominant)                              | 20 (83%)           | 11 (48%)            | 2 (25%)         |         |
| Type 2 (extramotor)                                     | 4 (17%)            | 12 (52%)            | 6 (75%)         |         |
| Braak NFT stage, median(25th, 75th percentile)          | 1 (0, II)          | II (II, III)        | II (II, III)    | <0.001  |
| Braak 0                                                  | 11 (46%)           | 3 (13%)             | 0 (0%)          |         |
| Braak I                                                  | 7 (29%)            | 7 (30%)             | 3 (38%)         |         |
| Braak II                                                 | 6 (25%)            | 8 (35%)             | 3 (38%)         |         |
| Braak III                                                | 0 (0%)             | 5 (22%)             | 2 (25%)         |         |
| Thal amyloid phase median (25th, 75th percentile)       | 0 (0, 1)           | 1 (0, 1)            | 0 (0, 2)        | 0.12    |
| Braak 0                                                  | 16 (67%)           | 9 (39%)             | 4 (50%)         |         |
| Braak I                                                  | 6 (25%)            | 9 (39%)             | 0 (0%)          |         |
| Braak II                                                 | 1 (4%)             | 2 (8%)              | 2 (25%)         |         |
| Braak III                                                | 1 (4%)             | 3 (13%)             | 2 (25%)         |         |
| Lewy body disease                                       |                    |                     |                 | 0.09    |
| BLBD                                                     | 1 (4%)             | 2 (8%)              | 2 (25%)         |         |
| TLBD                                                     | 1 (4%)             | 0 (0%)              | 0 (0%)          |         |
| DLBD                                                     | 0 (0%)             | 0 (0%)              | 1 (6%)          |         |
| Hippocampal sclerosis                                   | 0 (0%)             | 1 (4%)              | 0 (0%)          |         |
| Saito AGD stage                                          |                    |                     |                 | 0.32    |
| Stage 0                                                  | 21 (88%)           | 20 (87%)            | 6 (75%)         |         |
| Stage 1                                                  | 3 (13%)            | 1 (4%)              | 1 (6%)          |         |
| Stage 2                                                  | 0 (0%)             | 2 (8%)              | 1 (6%)          |         |

Abbreviations: AGD, argyrophilic grain disease; BLBD, brainstem type Lewy body disease; DLBD, diffuse Lewy body disease; TLBD, transitional (limbic) type Lewy body disease.

FIGURE 2  Representative images of TDP-43 lesions immunohistochemistry for pTDP43. Neuronal cytoplasmic inclusions are observed in the amygdala (A), dentate gyrus (B), CA1 (C), subiculum (D), entorhinal cortex (E), white matter of the parahippocampal gyrus (F), frontal cortex (G), and motor cortex (H). Scale bars = 100 μm in A–G
young-ALS, 65 ± 4 years in middle-ALS, and 76 ± 5 years in old-ALS. Time from first weakness to death was not statistically significantly different: 46 ± 28 months in young-ALS, 39 ± 25 months in middle-ALS, and 34 ± 29 months in old-ALS. The male-to-female ratio and score of last ALS-CBS were not different between the three groups.

3.2 | Pathologic features

The pathologic features of all cases are summarized in Table 2. The brain weight was significantly lower in old-ALS (1135 ± 199 g) than in young-ALS (1286 ± 117 g, \( p = 0.03 \)). Nishihira classification of TDP-43 pathology in ALS was significantly different between young-ALS and middle-ALS, with less type 2 (i.e., nonmotor TDP-43 pathology), in young-ALS (\( p < 0.05 \)). Braak NFT stage was significantly higher in old-ALS and middle-ALS compared with young-ALS group (II vs. I, \( p < 0.05 \)). Thal amyloid phase, frequency of Lewy body disease, frequency of HpScl, and frequency of AGD were not different between old-ALS, middle-ALS and young-ALS.

3.3 | TDP-43 immunohistochemistry

TDP-43 pathology was observed in all cases in motor cortex, hypoglossal nucleus, and anterior horn cells of the
spinal cord. The amygdala had NCIs as well as sparse DN (Figure 2A). NCIs were common in the dentate gyrus (Figure 2B), CA1 (Figure 2C), subiculum (Figure 2D), and entorhinal cortex (Figure 2E). The white matter of the parahippocampal gyrus was mildly affected in some cases with sparse GCI (Figure 2F). The middle frontal cortex was mildly affected in some cases, with sparse NCI and DN that did not show preferential distribution to any cortical layer (Figure 2G). The motor cortex had NCI, including some in Betz cells, as well as DN (Figure 2H). There were variable GCI in subcortical white matter. In extramotor areas, TDP-43 pathology was more frequent in old-ALS than in young-ALS and middle-ALS in the following regions: amygdala (100% vs. 29% and 57%; p < 0.01), dentate gyrus (75% vs. 13% and 52%; p < 0.01), CA1/subiculum (63% vs. 8% and 44%; p < 0.01), and entorhinal cortex (75% vs. 21% and 48%; p = 0.02). The frequency of TDP-43 pathology was not statistically different in middle frontal cortex between old-ALS, middle-ALS and young-ALS (38%, 38%, and 35%; p = 1). Comparing old-ALS, young-ALS and middle-ALS, the frequency of TDP-43 pathology was similar in the motor cortex. The morphology of TDP-43 pathology in the amygdala, dentate gyrus, CA1/subiculum, and entorhinal cortex was mostly preinclusions and NFT-like inclusion in all groups, and the difference in morphology of TDP-43 pathology was not evident between the three groups. Two cases had only subpial astrocytic processes associated with corpora amylacea in the amygdala (Figure 3).

3.4 | Quantitative digital image analysis and double-labeling immunofluorescence staining

We quantified the burden of TDP-43 pathology with digital image analysis. As shown in Figure 4, old-ALS cases had greater TDP-43 burden in the amygdala, dentate gyrus, subiculum and entorhinal cortex compared with young-ALS and middle-ALS. The TDP burden in the motor cortex was higher in old-ALS than in middle-ALS. In contrast, the TDP-43 burden was sparse in white matter of the parahippocampal gyrus and middle frontal cortex of almost all cases and did not differ between the three groups.

Of note, we found two outliers with respect to TDP-43 burden in the amygdala and dentate gyrus. In Case 1, TDP-43 pathology observed outside of the pyramidal motor system included middle frontal gyrus, hippocampus and amygdala. Case 2 had TDP-43 pathology in motor system, hippocampus, and amygdala. In typical ALS, medial temporal lobe structures are usually the last
regions affected [7]; therefore, the latter case is an exception to typical ALS staging. Case 2 had Braak NFT stage III as well as AGD, while Case 1 had Braak NFT stage II. To investigate the relation between TDP-43 and tau pathology, immunofluorescence double-staining with phospho-tau and pTDP-43 was performed in the amygdala. Colocalization of pTDP-43 and phospho-tau was observed in only a small subset of pretangles in Case 2 (data not shown).

4 | DISCUSSION

In this study, we compared the burden of TDP-43 pathology and clinical features of ALS in three age groups. The burden of TDP-43 pathology was greater in old-ALS than in young-ALS and middle-ALS in the limbic system. In contrast, we did not find a significant difference in clinical features, such as disease duration and cognitive function assessed by ALS-CBS, which is probably due to the small sample size of our cohort, the high variability of the disease, and the fact that we excluded patients with significant ADNC. These patients would have had a low score of ALS-CBS and would likely to have been included in the old-ALS group; therefore, the ALS-CBS score of this group was higher. Indeed, the average CBS score of 15.3 in our cohort was higher than the score of 14.5 in our previous study based on a large size clinical cohort [31].

Aging-associated processes, such as genetic instability, telomere attrition, epigenetic alterations, mitochondrial dysfunction, and cellular senescence may influence neurodegenerative disorders in the elderly, including ALS [8, 33, 34]. Accumulating evidence suggests that there are differences in clinical, pathologic, and genetic features, as well as the frequency of copathologies, between older and younger patients for a number of neurodegenerative disorders. For example, AD has been neuropathologically classified into three subtypes; typical AD, hippocampal-sparing AD, and limbic-predominant AD [35]. Hippocampal-sparing AD is more common in young AD individuals, and it is associated with atypical clinical presentations as well as a weaker association with APOE e4, whereas older AD individuals tend to have limbic-predominant AD, which is associated with amnestic clinical presentation and strong association with APOE e4 [35]. In genetically confirmed and sporadic FTLD-TDP, elderly patients tend to have limbic-predominant TDP-43 pathology and often an amnestic rather than frontal-behavioral clinical presentation [16]. These findings suggest that age may affect clinical phenotypes and pathologic features, including distribution and severity of pathology. Our results suggest that the distribution of TDP43 in ALS differs with age, and that TDP43 pathology in limbic regions may be a feature of ALS in the elderly.

A possible explanation for the limbic-predominant TDP-43 pathology in old-ALS is that it represents a concurrent age-related process, such as LATE. The differentiation of LATE from FTLD-TDP in the elderly remains controversial [36]. Robinson et al. suggested that the severity of TDP-43 burden in middle frontal cortex could be used to distinguish LATE from FTLD-TDP [37]; however, the difference observed may have been influenced by the young age of their patients with FTLD-TDP. FTLD-TDP in the elderly not only has a limbic predominance, but also less TDP-43 burden in frontal and temporal neocortices, making it difficult to distinguish it from LATE [16]. The possibility that both primary TDP-43 proteinopathy and LATE may coexist has not been specifically considered in previous reports. Until specific markers are found that can differentiate LATE from FTLD-TDP, this question will remain unresolved.

LATE may include both TDP-43 pathology associated with ADNC or HpScI [15]. Given that no patients in the old-ALS had HpScI, it is reasonable to assume that TDP-43 pathology in old-ALS may be associated with ADNC. Although we excluded cases with Braak IV-VI and Thal 4-5 to avoid strong influences of ADNC on TDP-43 pathology, the median Braak NFT stage was still higher in old-ALS than in young-ALS. Staging of TDP-43 pathology in AD has been proposed, in which TDP-43 pathology begins in limbic regions, such as amygdala (Stage 1), and subsequently affects entorhinal cortex (Stage 2) and subiculum (Stage 2) [38]. At present, it is not possible to exclude the possibility that greater TDP-43 pathology in limbic regions in old-ALS is due to LATE. The morphology of TDP-43 pathology in the limbic regions was not different between the three groups. It is worth noting that TDP-43-positive subpial processes, which are the most frequent pattern of TDP-43 pathology in the amygdala in LATE-NC [24, 39], was rarely seen in ALS. These findings suggest that TDP-43 lesions in the limbic regions in ALS may not simply be a complication of LATE.

TDP-43 pathology is frequent in AD, but the direct link between ADNC and TDP-43 pathology is not fully understood [40, 41]. A subset of patients with LATE have codeposition of TDP-43 and tau in neurons with NFTs [15, 42, 43]. Recent studies suggest that there may be mechanistic links between tau and TDP-43 pathologies. TDP-43, as an mRNA binding protein, suppresses tau mRNA and thus promotes tau mRNA instability by binding to the 3′-untranslated region of tau mRNA [44]. Other studies suggest that TDP-43 may regulate the ratio of 4 repeat-tau and 3 repeat-tau by binding to pre-mRNA intron 9 in MAPT gene [45]. In any event, evidence suggests that cytotoxicity of TDP-43 and tau are synergistic [46].

Clinicopathological correlations of TDP-43 pathology have been reported in ALS. Nishihira et al. concluded that Type 2 ALS, which has extramotor TDP-43 pathology, is associated with cognitive impairment. In contrast to these observations, in the current study we did not find greater frequency of cognitive impairment in
Type 2 ALS [6]. A possible limitation of this study is that by excluding ALS cases with moderate or marked ADNC, we may have underestimated the frequency of extramotor TDP-43 pathology in old-ALS since there is clearly a strong association between TDP-43 and both early and late-onset AD [15, 38, 40], which is observed in influencing this study; however, copathology increases with age, and copathology may influence pathology and clinical phenotype of ALS. In conclusion, the present study found differences in TDP-43 burden in limbic structures between old-ALS and young-ALS or middle-ALS. This finding suggests that the amygdala and hippocampus are vulnerable to TDP-43 pathology in old-ALS, possibly due to concurrent LATE or to TDP-43 pathology associated with ADNC, in particular NFTs. It is well known that the elderly may have multiple concurrent pathologic processes and that the limbic lobe structures are particularly vulnerable [16, 35, 47]. This study shows that this is also true for a neurodegenerative disorder that primarily affects the pyramidal motor system.

**AUTHOR CONTRIBUTIONS**

Aya Murakami: Study concept and design; acquisition, analysis, and interpretation of data; execution of the statistical analysis; drafting of the manuscript. Shunsuke Koga: Study concept and design; analysis and interpretation of data; execution of the statistical analysis; an edit of the manuscript; review and critique. Hiroaki Sekiya: Acquisition and interpretation of data; review. Keith A. Josephs: Study concept and design; review and critique. Dennis W. Dickson: Study concept and design; acquisition and interpretation of data; and critique. Leonard Petrucelli: review and provision of critical reagents. Bjorn Oskarsson: clinical characterization of the ALS cohort; review and critique. Kevin Boylan: clinical characterization of the ALS cohort.

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