

Research article

Phosphorylated NF- κ B subunit p65 aggregates in granulovacuolar degeneration and neurites in neurodegenerative diseases with tauopathy

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ABSTRACT

Granulovacuolar degeneration (GVD) was originally reported in Alzheimer's disease (AD) and later found in aging brains. Pathologically, GVD is thought to be associated with the development of tauopathy, but the precise mechanism remains unknown. Previous studies have suggested that GVD contains proteins associated with an inflammatory signal. In this study, we examined phosphorylated p65 (pp65), which is the activated form of a subunit of nuclear factor-kappa B (NF- κ B), in the hippocampus of 21 autopsied cases, including AD, amyotrophic lateral sclerosis cases with optineurin mutation (ALS-OPTN), and a variety of other neurodegenerative disorder cases and normal controls. In all cases, GVDs were immunopositive for pp65. The density of pp65-positive GVDs statistically correlated with that of casein kinase 1 delta (CK1 δ), which is known as GVD marker. pp65 was also detected in neurites in AD and ALS-OPTN. The number of neurons with pp65-immunoreactive GVD was significantly higher in the AD group than in the non-AD group. Double immunostaining showed the colocalization of CK1 δ and pp65. pp65-positive GVD was found in a neuron with AT8-positive neurofibrillary tangles. Moreover, pp65 was also found in neurites that were immunostained with phosphorylated tau, phosphorylated α -synuclein, or TDP-43 (transactivation response element DNA-binding protein 43 kDa). Therefore, the activation of the NF- κ B pathway may be related to the pathology of GVD formation and dementia with tauopathy, including AD and ALS-OPTN. We propose that pp65 is useful as a GVD marker, and that the NF- κ B pathway could be a therapeutic target not only for AD, but for age-related neurodegenerative diseases in general.

1. Introduction

Granulovacuolar degeneration (GVD) is defined by more than two double membrane-bound cytoplasmic vacuoles containing an electron-dense granule in a neuron [1]. GVD was originally observed in the hippocampal pyramidal neurons of Alzheimer's disease (AD) [1], and also in other AD brain regions [2]. Later, GVD was found in other adult-onset dementias [3], and also seen as an aging-induced change [1]. GVD is also thought to facilitate tauopathy [1], but the cellular

mechanisms of GVD remain unknown. It is also not known whether GVD formation precedes tangle formation, thus representing a pre-tangle, as suggested by Köhler [1], or is a separate but co-occurring pathology in its own right as suggested by Bondareff et al. [4].

A number of molecules are reported to exist in GVDs, including cytoskeletal and autophagy-related proteins [1]; several proteins involved in signal transduction pathways related to cell stress, such as the stress-activated protein kinase/c-Jun N-terminal kinase (p-SAPK/JNK) pathway [3]; and apoptosis-related proteins such as caspase 3 [5].

Abbreviations: GVD, granulovacuolar degeneration; AD, Alzheimer's disease; NF- κ B, nuclear factor-kappa B; ALS-OPTN, amyotrophic lateral sclerosis cases with optineurin mutation; CK1 δ , casein kinase 1 delta; p-SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; TNF- α , tumor necrosis factor- α ; IL1 β , interleukin-1 β ; pp65, phosphorylated p65; CERAD, consortium to establish a registry for Alzheimer's disease; PBS, phosphate-buffered saline; CA1, Cornet d'Ammon 1; HE, hematoxylin-eosin; NFT, neurofibrillary tangle; LC3, light chain 3

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Several recent studies also reported the upregulation of various nuclear factor-kappa B (NF- κ B) pathway activating factors, including tumor necrosis factor- α (TNF- α) [6] and interleukin-1 β (IL1 β), in AD [7].

These previous studies suggest that GVDs form under stress signals carried by NF- κ B pathway activation factors. However, the relationships among GVD, hippocampal degeneration due to tauopathy, and the NF- κ B pathway remain unknown. Additionally, to our knowledge, little is known about the distribution and expression of p65 in human tissues.

Here we report that phosphorylated p65 (pp65) aggregates in GVDs and neurites in the hippocampal area of ALS-OPTN, AD, and other neurodegenerative cases. Accordingly, we propose that the activation of NF- κ B may contribute to GVD in hippocampal degeneration.

2. Materials and methods

The study was approved by the ethics committee of Kyoto University (approval number R1038).

2.1. Subjects

Consecutive autopsies examined at the Kyoto University Hospital between 1988 and 2014 were studied. Pathologically and clinically diagnosed AD cases were chosen together with age-matched non-AD cases, including cases with and without tau pathology. The study investigated 21 subjects, including seven cases of AD, two cases of ALS-OPTN, nine cases with a variety of other neurodegenerative disorders, and three control cases without evidence of clinical dementia or movement disorders (Table 1). All cases were clinically and neuropathologically assessed and evaluated with Braak and Braak staging [8] and CERAD (Consortium to Establish a Registry for Alzheimer's disease) plaque score [9]. CK18 immunopositive GVDs were assessed by the GVD staging system proposed by Thal [2]. The pathological diagnosis of AD was made by the CERAD protocol [9]. The staging results are summarized in Table 1.

Table 1
Neuropathological and clinical information about the case series.

Case no.	Sex	Diagnosis	Age at death (years)	Braak stage	CERAD	GVD stage	PMI (hours)
1	M	AD	85	V	C	4	nd
2	F	AD	94	V	C	3	27
3	M	AD	93	V	C	5	5
4	F	AD	82	V	C	4	nd
5	F	AD	81	V	C	5	2
6	F	AD	89	V	C	3	21
7	F	AD PD	76	IV	C	3	5
8	M	PD	76	III	0	1	3
9	F	PSP	67	III	B	2	1
10	M	PSP	66	III	B	1	5
11	M	PSP	82	I	0	2	11
12	M	PSP	73	II	0	2	nd
13	M	CBD	78	II	A	2	13
14	M	DLB	76	III	0	1	1
15	M	ALS	65	0	0	1	1
16	M	ALS	82	III	0	2	2
17	F	ALS-OPTN	66	II	A	1	nd
18	F	ALS-OPTN	52	III	A	2	nd
19	M	control	72	II	0	1	nd
20	F	control	70	I	0	1	4
21	M	control	68	I	0	0	nd

Abbreviations: CERAD, consortium to establish a registry for Alzheimer's disease; GVD, granular vascular degeneration; PMI, postmortem interval; M, male; F, female; AD, Alzheimer's disease; PD, Parkinson's disease; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; DLB, dementia with Lewy bodies; ALS, amyotrophic lateral sclerosis with optineurin mutation; ALS-OPTN, amyotrophic lateral sclerosis with optineurin mutation; nd, not determined.

2.2. Immunohistochemistry

6- μ m thick sections from formalin-fixed, paraffin-embedded brain tissue were deparaffinized, and antigen-retrieved by autoclaving for 20 min at 120 °C using the Histofine deparaffinization and antigen retrieval buffer, at pH 6 (Product Code 415281; Nichirei, Tokyo, Japan), followed by overnight incubation at 4 °C with primary antibodies in phosphate-buffered saline (PBS) containing 3% bovine serum albumin.

Immunohistochemical staining was conducted with the peroxidase polymer-based method, using the Histofine Simple Stain MAX-PO Kit (Nichirei) and the DAB Substrate Kit (SK 4100, Vector Laboratories, Burlingame, CA, USA). The primary antibodies used were as follows: Rabbit polyclonal S536 phosphorylated p65 (pp65), (1:500, 600-401-265, Rockland Immunochemicals Inc., Gilbertsville, PA), mouse monoclonal casein kinase 1 delta (CK18) (1:2000, AF12G4, ab85320, Abcam, Cambridge, MA, USA), mouse monoclonal hyperphosphorylated tau (1:500, AT8 clone, No. 90206; Innogenetics, Ghent, Belgium), mouse monoclonal phosphorylated α -synuclein (1:2000, clone #64, No. 015-25191; Wako Chemicals, Richmond, VA), mouse monoclonal phosphorylated TDP-43 (pTDP-43, 1:2000, No. TIP-PDT-M01; Cosmo Bio Co., Ltd., Tokyo, Japan), and mouse monoclonal amyloid beta (A β) pretreated with formic acid for 5 min (1:400, clone 6E10, No. 803001; BioLegend, San Diego, CA).

2.3. Semiquantitative analysis of immunolabeled neurons and data analysis

The number of CK18 and pp65 immunopositive neurons (with more than two immunopositive granules) was counted in the Cornet d'Ammon 1 (CA1) region of the hippocampus using light microscopy at 20x magnification. The CA1 area was measured using Image J (version 1.50i, Wayne Rasband, NIH, USA). The density of immunopositive neurons was computed for each case.

Data are expressed as means $\pm$ standard deviation (SD). The Pearson correlation coefficient was used to assess the correlation between CK18 density and the number of pp65 immunopositive neurons. For comparisons between AD and non-AD groups, the Mann-Whitney test was performed. The density of pp65-immunopositive neurons and Braak and Braak stage were analyzed by Spearman rank-order correlation coefficient analysis. Statistical analysis was performed using SPSS, version 20.0 (SPSS Inc., Chicago, IL, USA), and statistical significance was set at $p < 0.05$.

2.4. Immunofluorescence microscopic study

After paraffin removal and antigen retrieval, the sections were incubated overnight at 4 °C in a cocktail of primary antibodies (pp65, CK18, AT8, phosphorylated α -synuclein, pTDP-43, and A β). Sections were subsequently rinsed with PBS and incubated for an additional 90 min at room temperature in a cocktail of secondary antibodies: goat anti-mouse and goat anti-rabbit Alexa Fluor (1:200, Molecular Probes, Eugene, OR, USA). Sections were washed and coverslipped with VECTASHIELD (Vector Laboratories) and observed with a Fluoview FV-1000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

3. Results

3.1. Immunohistochemistry

This study included AD, non-AD dementia cases and non-dementia control cases (Table 1). Brains from autopsied cases were immunostained with anti-pp65 and anti-CK18 antibodies. In hematoxylin-eosin (HE) staining of AD cases, GVD was abundantly found in the hippocampus (Fig. 1a), and the CK18 antibody also immunostained GVDs, as previously reported, in each case (Fig. 1b, d, g, j).

In the AD cases, pp65-positive round granules (Fig. 1c, h) were found within a large number of hippocampal pyramidal neurons. In

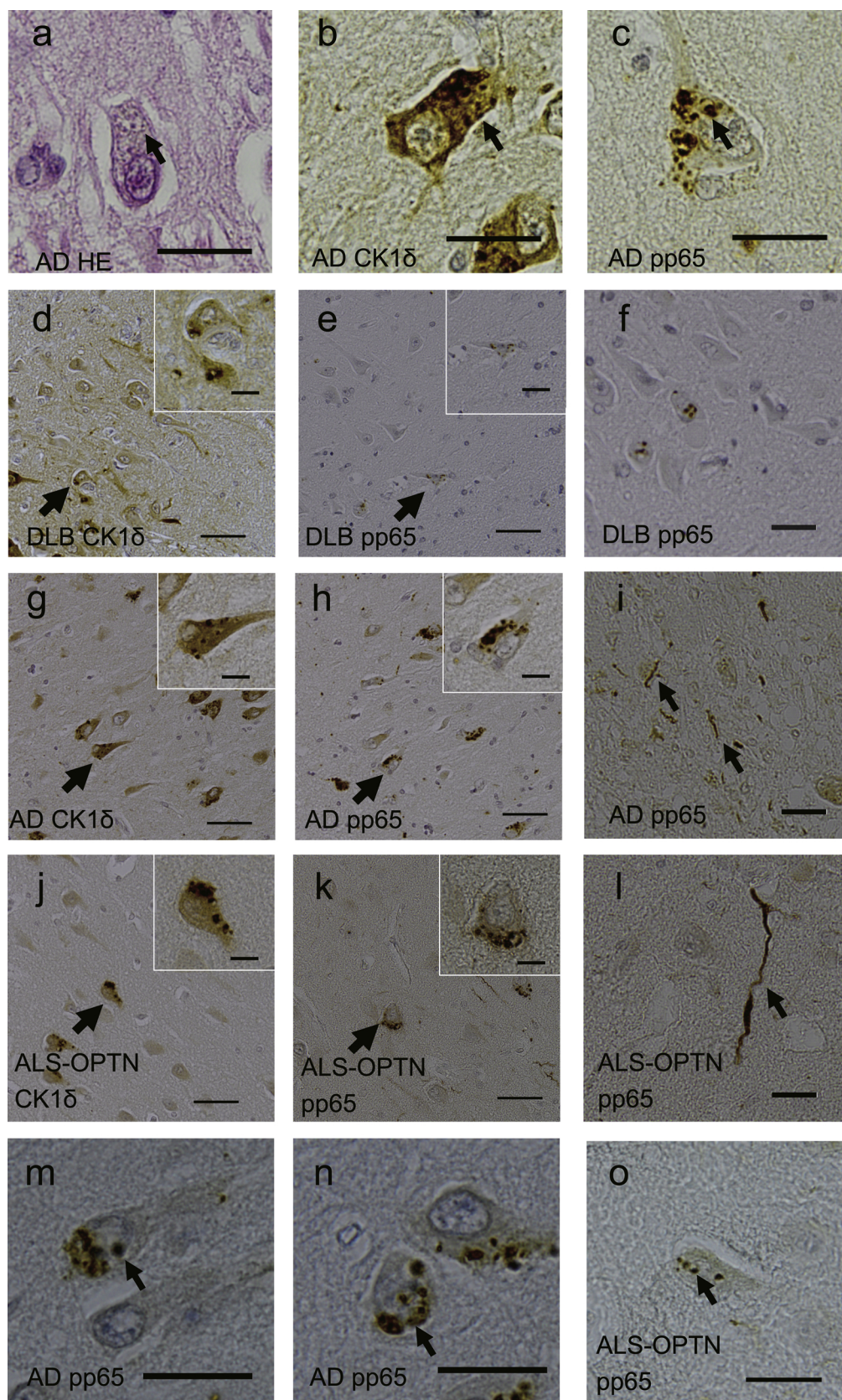


Fig. 1. In Hematoxylin-Eosin staining of an Alzheimer's disease (AD) case, typical granular vascular degenerations (GVDs) were found in the hippocampus (a). GVD in an AD case immunopositive for Casein kinase 1 delta (CK1δ) (b). Similar granular cytoplasmic inclusions, positive for phosphorylated p65 (pp65), were found in neurons (c). The arrows in each picture (a, b, c) indicate a typical GVD, consisting of a granular component and a surrounding vacuolar component.

pp65-immunoreactive granular cytoplasmic inclusions were found in neurons in a non-AD case (e), an AD case (h), and an amyotrophic lateral sclerosis with optineurin mutation (ALS-OPTN) case (k) in the CA1 region. The distribution and morphological features of pp65-positive inclusions were similar to those of CK1δ-positive GVDs (d, g, j). The inset shows the magnification of the neurons denoted by an arrow in each picture (d, e, g, h, j, k).

pp65 was also positive in some short neurites in AD (i, arrow). Long neurites were immunostained with pp65 antibody in a case of ALS-OPTN (l, arrow). pp65 was observed inside or peripheral to the nucleus in some neurons in AD (m, n) and ALS-OPTN (o) cases.

Scale bars: a, b, c; 25 μm, d, e, g, h, j, k; 50 μm (inset; 12.5 μm), f, i, l; 25 μm.

some AD cases, similar inclusions were found in the neocortex. The typical diameter of a pp65-positive granule was 1 μm, and the diameter of a pp65-negative vacuole approximately 5 μm (Fig. 1c). The morphology of the pp65-positive inclusions (Fig. 1c) was similar to that of

GVDs in HE staining (Fig. 1a) and CK1δ-immunopositive GVDs (Fig. 1b). They were compatible with the previously reported morphological features of GVDs [1]. The morphological features of pp65-positive granules were identical in the AD hippocampus and neocortex.

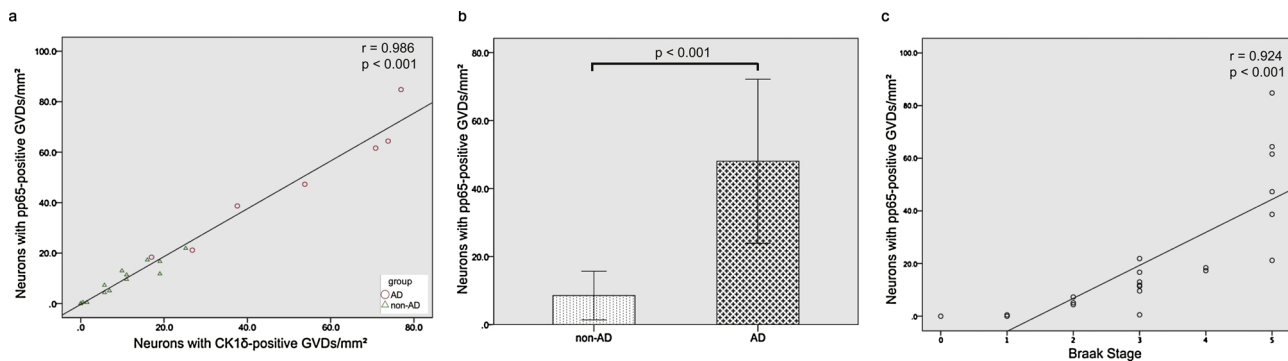


Fig. 2. Quantitative analysis of pp65-positive granular vascular degenerations (GVDs) in the hippocampal CA1 region. The analysis demonstrates the correlation between the number of neurons immuno-positive to phosphorylated p65 (pp65) and casein kinase 1 delta- (CK18) positive neurons (a). The density of neurons with pp65-immunoreactive GVDs in Alzheimer's disease (AD) cases was significantly higher than that in non-AD cases in the hippocampal CA1 region (b). The density of neurons with pp65-positive GVD was associated with the Braak and Braak stage group (c).

In other non-AD neurodegenerative cases (Fig. 1e) and in the ALS-OPTN cases (Fig. 1k), similar pp65-positive granules were also found, especially within the CA1 region of the hippocampus, but were confined to the hippocampus and not found in the neocortex. The morphological features and distribution of pp65-positive granules were similar to those of CK18-positive GVDs in each case (Fig. 1d, g, j).

In AD cases, short neurites in the neuropil were also immunostained with the anti-pp65 antibody (Fig. 1i), while the anti-CK18 antibody only recognized GVDs. In the ALS-OPTN cases, long neurites were frequently immunostained with the anti-pp65 antibody in the hippocampus (Fig. 1l). pp65 was also observed inside or peripheral to the nucleus in some neurons in AD (Fig. 1m, n) and ALS-OPTN (Fig. 1o).

In summary, GVD was immunostained by the pp65 antibody to the same degree as CK18, which is known as one of the GVD markers. This trend was constant, regardless of the underlying diseases.

3.2. Semiquantitative study

The density of neurons with pp65-positive inclusions was quantitatively correlated with that of CK18-positive GVD bodies (Fig. 2a). Quantitative analysis showed that the density of neurons with pp65-immunoreactive GVDs in AD cases was significantly higher than that of non-AD cases in the hippocampal CA1 region (Fig. 2b). In the hippocampus, pp65-immunoreactive GVD density correlated with Braak and Braak stages, as revealed by Spearman correlation analysis (Fig. 2c).

3.3. Immunofluorescence study

Double immunofluorescent staining with the CK18 and pp65 antibodies showed the co-localization of pp65 and CK18 in GVDs (Fig. 3a–d). In some of the AD neurites, pp65 was partially co-localized with AT8 (Fig. 3e–h). The AT8 antibody immunostained neurites more abundantly than the pp65 one (Fig. 3e–h). Some phosphorylated α -synuclein (pSyn)-positive neurites were immunopositive to the pp65 antibody (Fig. 3m–p).

In the AD cases, pp65-positive GVD was also found in neurons with AT8-immunopositive neurofibrillary tangles (NFTs). However, the AT8 antibody was rarely immunopositive in GVD (Fig. 3i–l). A β was immunopositive in the AD senile plaque, but immunonegative in GVDs. However, pp65 was positive in GVDs but negative in the senile plaque (Fig. 3q–t).

In the ALS-OPTN cases, a merged picture of the pp65 and pTDP-43 antibodies showed partial coexistence (Fig. 3x). The pp65 antibody immunostained neurites more abundantly than pTDP-43 (Fig. 3u, v). In the ALS-OPTN case, AT8 did not immunostain pp65-positive neurites (data not shown).

4. Discussion

In the present study, we showed that GVDs were immunostained by the pp65 antibody. The positive ratio of pp65-positive GVDs was similar to that of CK18, which is known as a GVD marker. pp65-immunopositive GVDs were abundantly found in the subiculum and CA1 zone of the hippocampus. This result is compatible with a previous study reporting that pyramidal neurons in the subiculum and CA1 zones of the hippocampus are most susceptible to GVD [1]. This trend was constant, regardless of the underlying disease. Our data also showed that pp65-positive GVD inclusions were found in about 60% of the hippocampus in aged individuals without dementia. We described the similarity of distribution and morphological features of pp65- and CK18-positive GVD. On the other hand, we did not evaluate the total frequency of merged stains. To investigate the significance of pp65 as a GVD marker, a comparison of pp65 with other GVD markers is needed.

Our results suggest that GVDs are formed under the chronic activation of the NF- κ B pathway. The most common NF- κ B heterodimer consists of p50 and p65 (RelA). The inactive form of p65 is present in the cytoplasm. When the NF- κ B pathway is stimulated by proinflammatory cytokines such as TNF- α [10] and IL1 β [11], p65 is translocated into the nucleus and activates various transcription factors. After activation, p65 is digested, terminating the activation of transcription factors. Inducible phosphorylation stabilizes p65, and positively regulates NF- κ B pathway activity [12]. Constant activation of the NF- κ B pathway could lead to neurodegenerative processes. Actually, the NF- κ B pathway is known to activate the caspase 3-dependent apoptosis pathway [13], and caspase 3 was also reported to exist in GVD [5]. Therefore, the presence of pp65 in GVD indicates the activation of the NF- κ B pathway and of an apoptotic process in GVD-positive neurons.

In the present study, pp65-positive GVD was significantly more frequent in the AD group than in controls, and associated to high levels of the Braak and Braak staging of tau protein. These findings indicate that the activation of the NF- κ B pathway is related to the pathology of GVD formation and to dementia with tauopathy, including AD.

GVD was reported to represent a late stage of autophagy [14]. In the process of autophagy, intact microtubules are required for the fusion of lysosomes or endosomes with autophagosomes [1]. Considering that tau plays an important role in stabilizing microtubules, the disruption of the microtubule network could mechanistically link the formation of GVD to tau pathology. The interpretation of the pathology is still controversial. Some studies imply that GVD contains pre-tangles and contributes to early NFT formation [15,16], which is in conflict with the findings of other studies that indicate that GVD can be considered as a separate process [4,17]. However, GVDs were reported to be less frequent in neurons bearing mature AT8-positive NFTs [15]. AT8 is a

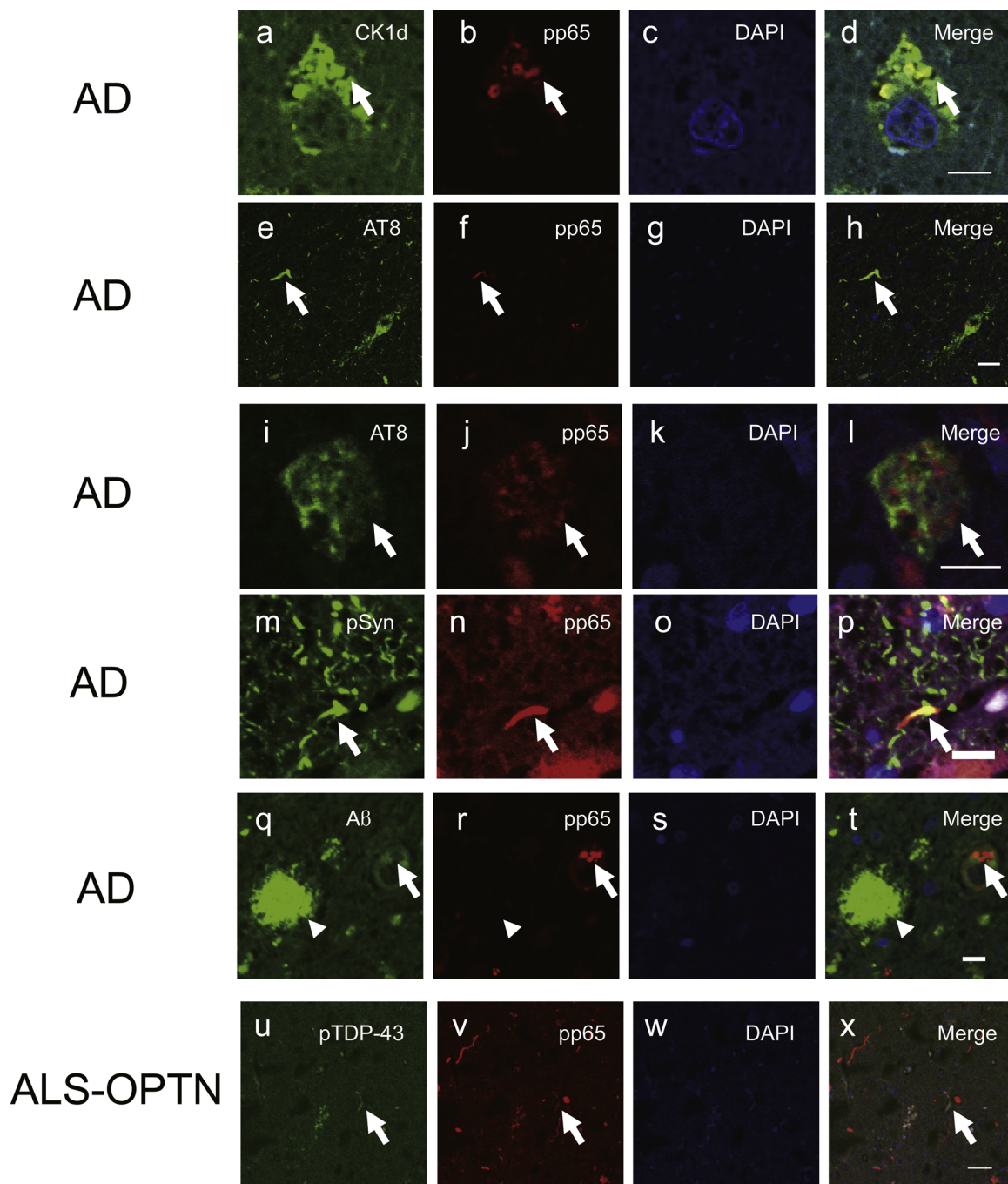


Fig. 3. Sections double-labeled with casein kinase 1 delta (CK1δ; a; green fluorescence) and phosphorylated p65 (pp65; b; red fluorescence) antibodies. The merged picture shows the overlap of green and red fluorescence, indicating co-localization of CK1δ and pp65 in granulovacuolar degeneration (GVD) bodies (d, arrow) in an Alzheimer's disease (AD) case.

pp65 was partially immunostained in an AT8-positive neurite (e–h, arrow). Double immunofluorescent staining of AT8 (i) and pp65 (j) showed AT8-positive NFT (i) and pp65-positive GVD (j) in the same neuron (i–l). AT8 was not immunostained in GVD (l). Some phosphorylated α-synuclein (pSyn)-positive neurites were immunopositive to the pp65 antibody (m–p). Amyloid β (Aβ) was positive in the senile plaque of AD (arrowhead, q), but negative in GVDs (arrow, r). On the other hand, pp65 was positive in GVDs (arrow, r), but negative in the senile plaque (arrowhead, q).

pTDP-43 (u) was partially positive in the pp65-positive (v) neurite in amyotrophic lateral sclerosis with optineurin mutation (ALS-OPTN) (u–x).

Scale bars: a–d; 10 μm, e–h; 25 μm, i–l; 10 μm, m–p; 10 μm, q–t; 10 μm, u–x; 50 μm.

phosphorylation-dependent monoclonal antibody commonly used as a marker of abnormal phosphorylation in the brain of Alzheimer's patients. Although several tau epitopes are detected in GVD granules, it was also reported that GVD granules were less frequently AT8-immunopositive [16]. Our immunofluorescence study showed that AT8 and pp65 exist in the same NFT-positive neuron, without co-localization. This finding might be consistent with the idea that GVD represents

an early stage of tau aggregation, in agreement with Köhler [1], but also with the concept of a separate but co-occurring pathology in its own right, as suggested by Bondareff et al. [4]. A limitation of our study is that the results are based on descriptive data. To determine the relationship between tauopathy, GVD, and pp65, future studies using longitudinal samples or *in vivo* studies should examine how NF-κB activation influences GVD formation.

While the most evident pp65-positive structure was GVD, neuropil threads also occasionally appeared. Much of the tau pathology lies within neuritic processes in the form of neuropil threads [18]. Synaptic changes were reported to precede neuronal loss in a variety of neurodegenerative diseases, including Alzheimer's [18], and synaptic dysfunction could be the main cause of transneuronal degeneration. Since abnormal neurite structures, such as neuropil threads and dystrophic neurites, are often found in the lesion where neuronal cell death occurs [18], the formation of abnormal neurites may signal neuronal death. Dystrophic neurites also contain autophagy-associated proteins, such as microtubule-associated protein light chain 3 (LC3) [19]. Given previous studies and our data, dystrophic neurites could share some pathogenic mechanisms with GVD, through the dysfunction of autophagy and the NF- κ B pathway.

Moreover, the pp65 antibody also coexisted with phosphorylated α -synuclein in neurites in the AD hippocampus. In the ALS-OPTN case, long neurites were frequently immunostained with the anti-pp65 antibody, and the pp65 antibody also coexisted with pTDP-43 in neurites in the hippocampus of ALS-OPTN cases. Recently we have reported tau pathology in ALS patients with OPTN mutation [20], and pp65 aggregation in the spinal cord in the same pathology [21]. Considering that OPTN dysfunction impairs the regulation of selective autophagy and of the NF- κ B pathway [22], our results are consistent with the idea of a relationship among GVD, tauopathy and the NF- κ B pathway. However, the number of cases with synucleinopathy and TDP-43 pathology was rather limited in our study, so that the precise role of NF- κ B in degenerative processes requires further study including proteinopathies other than tauopathy.

Given our results and those of previous studies, it is reasonable to assume that the NF- κ B pathway underlies the formation of GVD and synaptic changes in the hippocampus in degenerative diseases. This mechanism could be most evident in AD tauopathy.

Limitations of this study include the limited number of samples: To avoid selection bias, examination of pp65 in GVD requires further studies using larger samples of human brain tissues. Another limitation is that the present study is based on descriptive results. Future studies using larger and longitudinal samples should investigate how NF- κ B activation influences GVD formation.

Several recent reports suggest that AD patients show upregulation of various NF- κ B pathway activating factors, including TNF- α [6] and IL1 β [7]. In particular, TNF- α was reported to cause phosphorylation of tau *in vivo* [23]. Recently the effectiveness of anti-TNF- α antibody therapies has been reported in an AD mouse model [23]. Given that pp65-positive GVD was observed not only in AD, but also in other senile neurodegeneration cases, the NF- κ B pathway could also be a therapeutic target for other senile neurodegenerative diseases.

5. Conclusions

pp65 was abundantly found in GVDs, and we propose that the activation of NF- κ B may contribute to GVD formation and hippocampal tauopathy with dementia.

Declarations of interests

The authors declare no conflict of interest.

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Contribution

"Each author is required to declare his or her individual contribution to the article: all authors must have materially participated in the

research and/or article preparation, so roles for all authors should be described. The statement that all authors have approved the final article should be true and included in the disclosure.

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