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Increased Expression of C/EBP Homologous Protein, a Marker of Endoplasmic Reticulum Stress, in the Brains of *App*$^{NL-G-F/NL-G-F}$ Knock-in Alzheimer's Disease Model Mice

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Alzheimer's disease (AD) is one of the most common types of progressive dementia. Recently, endoplasmic reticulum (ER) stress was suggested as a potential event involved in AD development. Thus, targeting ER stress may be an effective AD treatment. The involvement of ER stress in the brains of amyloid precursor protein knock-in AD model mice (*App*$^{NL-G-F/NL-G-F}$) with Swedish/Iberian/Arctic mutations found in human familial AD remains unclear. This study aimed to clarify whether the expression of ER stress marker C/EBP homologous protein (CHOP) was enhanced in the brains of *App*$^{NL-G-F/NL-G-F}$ AD model mice. Our immunofluorescence staining results showed that similar to the expression pattern of amyloid-β (Aβ), CHOP demonstrated an age-dependent increase in the numbers and sizes of spotted signals in the cerebral cortex and hippocampus of these 4- to 10-month-old AD model mice but not in their age-matched controls. These findings suggested that CHOP expression was upregulated in close association with Aβ expression, and that CHOP was involved in neuropathy caused by Aβ accumulation. Future investigations of the localization and variations in expression levels of other ER stress-related proteins in this mouse model using immunofluorescence staining will lead to a more detailed estimation of the relationship between ER stress and AD pathogenesis.

Key words Alzheimer's disease, amyloid-β, C/EBP homologous protein, *App*$^{NL-G-F/NL-G-F}$

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease that severely affects cognitive abilities, leading to memory loss. It is the prevalent cause of dementia in late adult life and is associated with high morbidity and mortality rates in older adults.[1)]

The neuropathological hallmarks of AD are amyloid-β (Aβ) plaques and neurofibrillary tangles formed by the intracellular accumulation of hyperphosphorylated tau protein.[2)] To date, only a few approved drugs have been used for the clinical treatment of AD, including cholinesterase inhibitors and NMDA receptor antagonists, which only relieve the symptoms or slow down disease progression and do not provide a cure.[3)] Based on the amyloid cascade hypothesis,[4)] the clearance of Aβ plaques from the brain may treat AD and halt disease progression, and this has promoted the development of innovative antibody drugs for preventing Aβ aggregation in the brain, such as lecanemab.[5)] However, these antibody drugs are indicated for patients with early AD[6)] and are unable to cure advanced cases.[3)] Therefore, a thorough investigation of the changes in the brain following Aβ accumulation is necessary to identify novel targets and subsequently develop effective AD treatments.

Endoplasmic reticulum (ER) stress was recently reported as a potential event in AD development, and several studies have suggested that Aβ accumulation-induced ER stress is involved in neurodegeneration in AD.[7,8)] The accumulation of unfolded/misfolded proteins within the ER lumen leads to ER dysfunction (ER stress).[9)] The unfolded protein response (UPR) activates and promotes the degradation of misfolded proteins and increases the ability to correct protein folding. However, under chronic or excessive ER stress, the UPR fails to maintain ER homeostasis, resulting in activation of the apoptosis signaling pathway, leading to the development of various diseases.[10)] ER-induced apoptosis occurs via three primary pathways: IRE1/ASK1/JNK, caspase-12 kinase, and C/EBP homologous protein (CHOP)/GADD153. The CHOP pathway plays a critical role in ER stress-induced apoptosis caused by neurological diseases.[11,12)] Therefore, ER stress is considered to act in a mediator-like role in AD pathology, and elucidating the relationship between ER stress and AD development could enable the identification of new therapeutic targets in AD treatment.

Mice with mutations inserted into the Aβ and tau genes,

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which are considered responsible for AD onset, have been used as models of memory impairment. Transgenic mouse models of AD include the Tg2576 model, in which amyloid precursor protein (APP) is overexpressed; the 5XFAD transgenic model, characterized by the overexpression of APP and presenilin 1 and the presence of mutations observed in familial AD; and the 3xTg model, with mutations leading to the overexpression of presenilin, APP, and tau. However, the AD pathology in these mouse models differs from the AD pathology in humans due to the overproduction of APP. Recently, Saito *et al*. generated a single APP knock-in mouse model, $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$.[13] In this model, the mutations observed in familial AD (Swedish, Beyreuther/Iberian, and Arctic mutations) were introduced into the endogenous mouse APP gene, leading to the development of progressive AD-related pathologies, including cognitive decline, Aβ deposition, and neuroinflammation. This model is considered to reflect the human condition without the artificial effects of APP overexpression.

The involvement of ER stress in AD development requires careful evaluation to enable the identification of effective prevention and treatment strategies. The detection of ER stress markers, including CHOP, in the brain of several different AD mouse models was the subject of a review by Hashimoto and Saido.[14] Although an enhanced ER stress response was observed in 5XFAD and 3xTg mouse models,[15,16] no ER stress response was observed in APP-single-Tg or APP knock-in mouse models, including $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$.[17] Because most of these reports were based on results derived from western blot analysis of total proteins extracted from the hippocampus and cerebral cortex, the target proteins might have been diluted, making it difficult to accurately detect increases or decreases in their expression. Thus, it is imperative that conclusions are not only based on the results of western blot analysis but also on the results of careful examination of the localization of Aβ and ER stress markers, such as CHOP, in the brains of AD model mice. Therefore, in this study, we used immunofluorescence staining to analyze CHOP expression in the brains of $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice. Additionally, we investigated whether CHOP expression was associated with the increased amyloid levels that have been observed with age.

MATERIALS AND METHODS

Animals The original lines of APP knock-in ($App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ and $App^{NL/NL}$) mice with a C57BL/6J genetic background were obtained from the RIKEN Center for Brain Science (Wako, Japan) and maintained by brother–sister mating at the Institute for Animal Experimentation, Toho University. In addition to the humanized mouse Aβ sequence, the Swedish familial AD mutation is inserted in $App^{NL/NL}$ knock-in mice and a triple mutation (Swedish, Beyreuther/Iberian, and Arctic familial AD mutations) is inserted in $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in mice. The $App^{NL/NL}$ knock-in mice were used as negative controls. Following weaning at postnatal days 28–35, all mice were housed socially in same-sex groups in a temperature-controlled environment (24 ± 2°C) under a 12-h light/dark cycle (lights on at 8:00, lights off at 20:00), with access to food and water *ad libitum*. The mice were tested at approximately 4–10 months of age.

All experiments were performed in accordance with the Toho University guidelines for animal care, handling, and termination, which comply with the international and Japanese guidelines for animal care and welfare (approval number: 21-31-476).

Brain Fixation While under anesthesia with a cocktail combination (medetomidine [0.3 mg/kg] + midazolam [4 mg/kg] + butorphanol [5 mg/kg]), the mice were intracardially perfused with phosphate-buffered saline (PBS, Wako Pure Chemical Industries, Osaka, Japan), followed by 4% paraformaldehyde solution (Nacalai Tesque, Inc., Kyoto, Japan). Then, the brains were collected and postfixed in 4% paraformaldehyde solution at 4°C overnight before storage in FD Tissue Storage Solution (FD NeuroTechnologies, Inc., MD, USA) at −20°C until tissue sectioning.

Immunofluorescence Staining of Aβ and CHOP Coronal sections of the brain (thickness, 50 μm) were prepared using a vibratome (Neo LinearSlicer MT, Dosaka EM Co., Ltd., Kyoto, Japan). Antigens were retrieved by soaking the sections in HistoVT One solution (Nacalai Tesque, Inc.) for 30 min at 70°C. After washing with PBS containing 0.3% Triton X-100, sections for Aβ staining were further incubated with 70% formic acid for 15 min. Then, all sections were blocked for 1 h at room temperature using BlockAid Blocking Solution (Thermo Fisher Scientific, Inc., MA, USA) containing 0.3% Triton X-100, followed by overnight incubation at 4°C with primary antibodies in the blocking buffer. The primary antibody used for Aβ staining was mouse anti-Aβ (specific for human Aβ1–16; 1:500; BAN50; Wako Pure Chemical Industries, Osaka, Japan), and mouse anti-DDIT3 (1:100; ab11419; Abcam plc., Cambridge, UK) and mouse anti-CHOP (1:100; L63F7; Cell Signaling Technology, Inc., MA, USA) were used to stain CHOP. DDIT3 is the gene name for CHOP and refers to the same.

After three washes with PBS, the sections were incubated for 1 h at room temperature in blocking buffer with the following secondary antibodies: Alexa Fluor 488 Goat anti-Mouse IgG1 (1:1000; A-21121; Thermo Fisher Scientific, Inc.) for anti-Aβ antibody, Alexa Fluor 488 Goat anti-Mouse IgG2b (1:1000; A-21141; Thermo Fisher Scientific, Inc.) for anti-DDIT3 antibody, and Alexa Fluor 594 Goat anti-Mouse IgG2a (1:1000; A-21135; Thermo Fisher Scientific, Inc.) for anti-CHOP antibody. After three washes with PBS, the immunostained slides were mounted with Prolong Glass Antifade Mountant with NucBlue Stain (Thermo Fisher Scientific, Inc.) and cured for 48 h. Immunofluorescence images of the sections were captured using a BZ-X700 fluorescence microscope (Keyence, Osaka, Japan). Multiple images were taken at 20× magnification and then joined together for quantification of the areas of Aβ plaques using WinROOF Education Version 2.4.0 (Mitani Corp., Tokyo, Japan).

RESULTS AND DISCUSSION

Changes in Aβ Plaque Staining in $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ Mice with Age $App^{NL/NL}$ mice are recombinants in which the mouse Aβ sequence is humanized and a Swedish familial AD mutation is inserted. In contrast, $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice have a humanized Aβ sequence and Swedish, Beyreuther/Iberian, and Arctic familial AD mutations, and Aβ deposition in the cortex of these model mice has been reported to begin by 2 months old and show saturation by the age of 7 months. Thus, we used $App^{NL/NL}$ mice as negative controls in our study.[13]

Figure 1 shows images of Aβ deposition in $App^{NL/NL}$ and $App^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ brains. Figure 1A shows the immuno-

fluorescence staining results for Aβ in the hippocampus and cerebral cortex of 8-month-old mice. Specific fluorescence derived from Aβ antibodies (arrows) was observed in *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ brains. Figure 1B shows the immunofluorescence staining results for Aβ at 4, 6, 8, and 10 months of age. Anti-Aβ antibody-derived fluorescence was observed in 4-month-old *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice, with particularly high expression in the cortex. Consistent with the literature[13], age-dependent progressive Aβ amyloidosis was observed in *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice, with increased deposition throughout the cortex at 8 months of age. The hippocampus region of the brain is involved in memory, and the slight accumulation of Aβ in the hippocampus was observed at 4 months of age, with increased deposition throughout the hippocampus at 10 months of age. Figure 1C shows the percentage of anti-Aβ antibody-derived fluorescent areas in the brains of *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice, including the hippocampus and cerebral cortex. Aβ aggregation markedly increased from the age of 6–8 months and continued to increase with age until the mice were 10 months old.

CHOP Identification in *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ Mice Brains We compared immunofluorescence staining images between 8-month-old *App*$^{NL/NL}$ and *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice to determine the expression of ER stress markers in mouse brains (Figs. 2A and 2B). First, we compared the immunofluorescence staining results obtained using two commercially available antibodies against CHOP. CHOP-derived luminescence was observed in *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ brains but not in *App*$^{NL/NL}$ brains. Furthermore, the staining patterns in the cerebral cortex and hippocampus were similar to those obtained using the Aβ antibody. Next, we compared the staining patterns between the two types of antibodies, revealing that CHOP antibody L63F7 was more sensitive compared with ab11419.

The CHOP antibody L63F7 used in this study was the same as that used in previous studies[17] examining CHOP expression by western blot analysis in *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ brains. Although these studies did not show increased CHOP expression in the brain total proteins of *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in AD model mice,[17] our study, which used an immunofluorescence staining method, showed a clear increase in CHOP expression in *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in AD model mice. As mentioned in the review by Hashimoto and Saido, the relationship between AD pathology and ER stress has been investigated using various AD mouse models, but no unified conclusion has been reached.[14] In our study, immunofluorescence staining revealed that CHOP expression patterns in the brains of *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice were similar to those of Aβ plaques.

Changes in CHOP Expression in *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ Mice with Age We observed changes in CHOP expression with age using the L63F7 antibody. Figure 3 shows the immunofluorescence staining results for CHOP in brains of 4- to 10-month-old *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice. In 4-month-old mice brains, CHOP expression was mainly observed in the cerebral cortex. Additionally, CHOP expression in the cerebral cortex increased until 8 months of age. In the hippocampus, clear CHOP expression was observed in 6-month-old mice brains, with a marked increase in expression until 8 months of age. Similar to

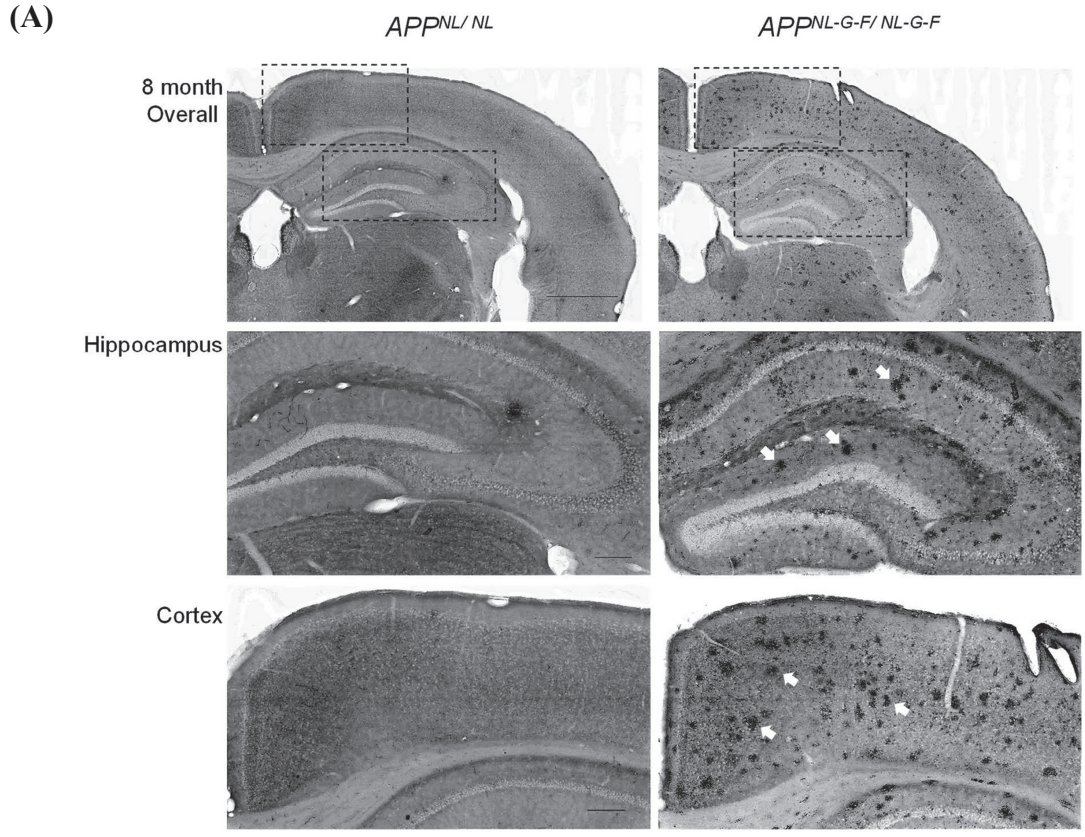
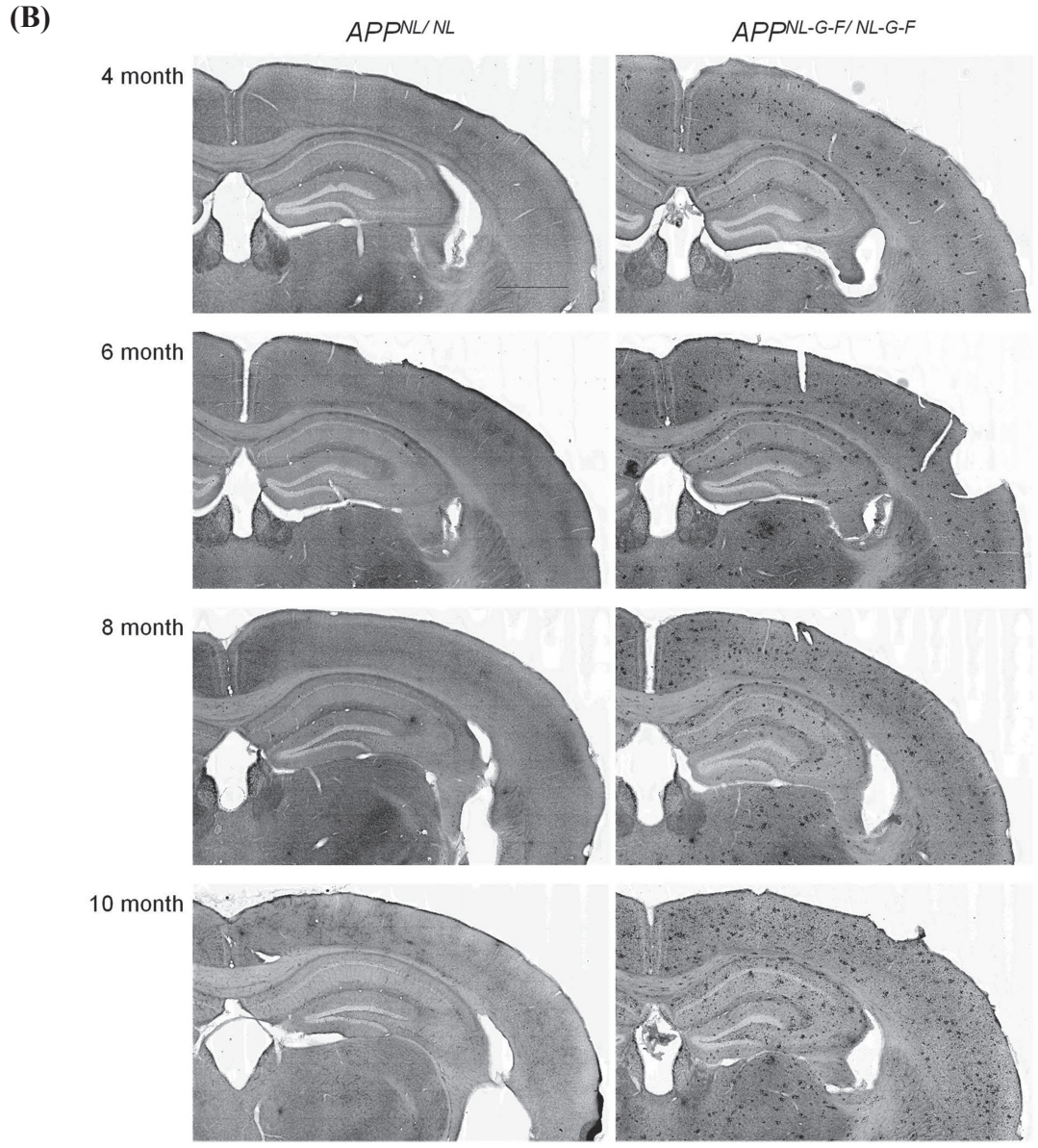


Fig. 1. Aβ Deposition in *App*$^{NL/NL}$ and *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ Mice Brains

(A) Immunofluorescence staining images of the hippocampus and cortex areas in the brains of 8-month-old *App*$^{NL/NL}$ and *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice using anti-Aβ antibody. The same magnification was used for each pair of photos. Scale bars represent 1,000 μm in the overall image and 200 μm in the images of the hippocampus and cortex.



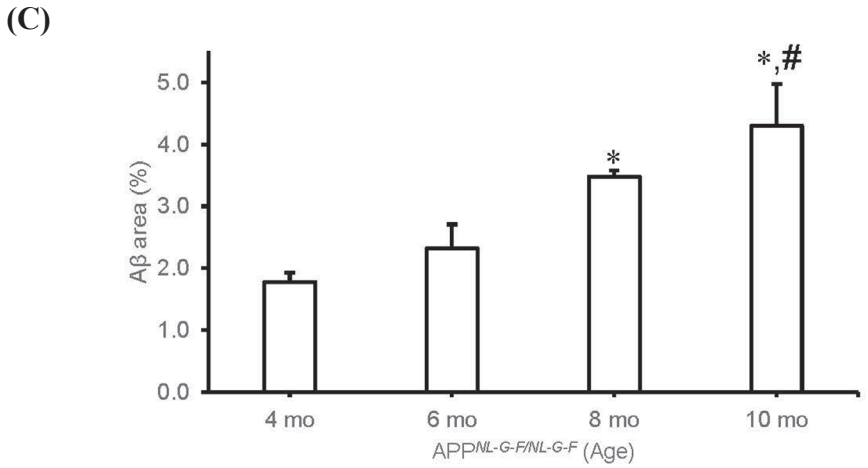


Fig. 1. (Continued)

(B) Immunofluorescence staining images of *App*$^{NL/NL}$ and *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice brains at 4, 6, 8, and 10 months old using anti-Aβ antibody. All photos have the same magnification. Scale bars represent 1,000 μm.

(C) Quantification of the ratio of the Aβ-positive areas. Data are presented as means ± standard deviations for each group (n = 3). *$p < 0.05$ versus 4 months, #$p < 0.05$ versus 6 months (Tukey multiple comparison test).

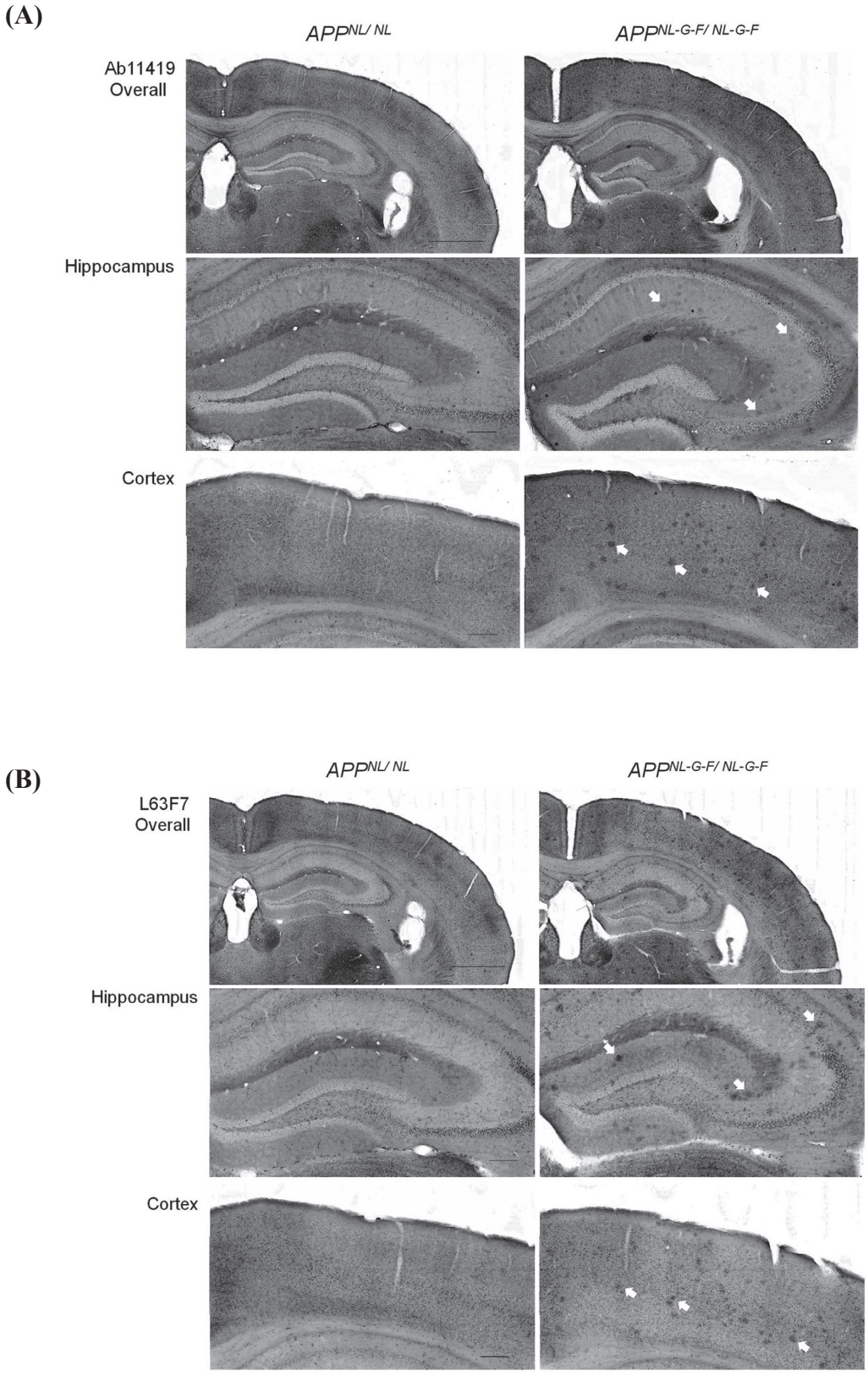


Fig. 2. Immunofluorescence Staining with Two Different Antibodies Against CHOP

(A) Immunofluorescence staining images of the hippocampus and cortex areas of 8-month-old *App*$^{NL/NL}$ and *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice using anti-DDIT3 antibody Ab11419. The same magnification was used for each pair of photos. Scale bars represent 1,000 μm in the overall image and 200 μm in the images of the hippocampus and cortex.

(B) Immunofluorescence staining images of the hippocampus and cortex areas of 8-month-old *App*$^{NL/NL}$ and *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice using anti-CHOP antibody L63F7. The same magnification was used for each pair of photos. Scale bars represent 1,000 μm in the overall images and 200 μm in the images of the hippocampus and cortex.

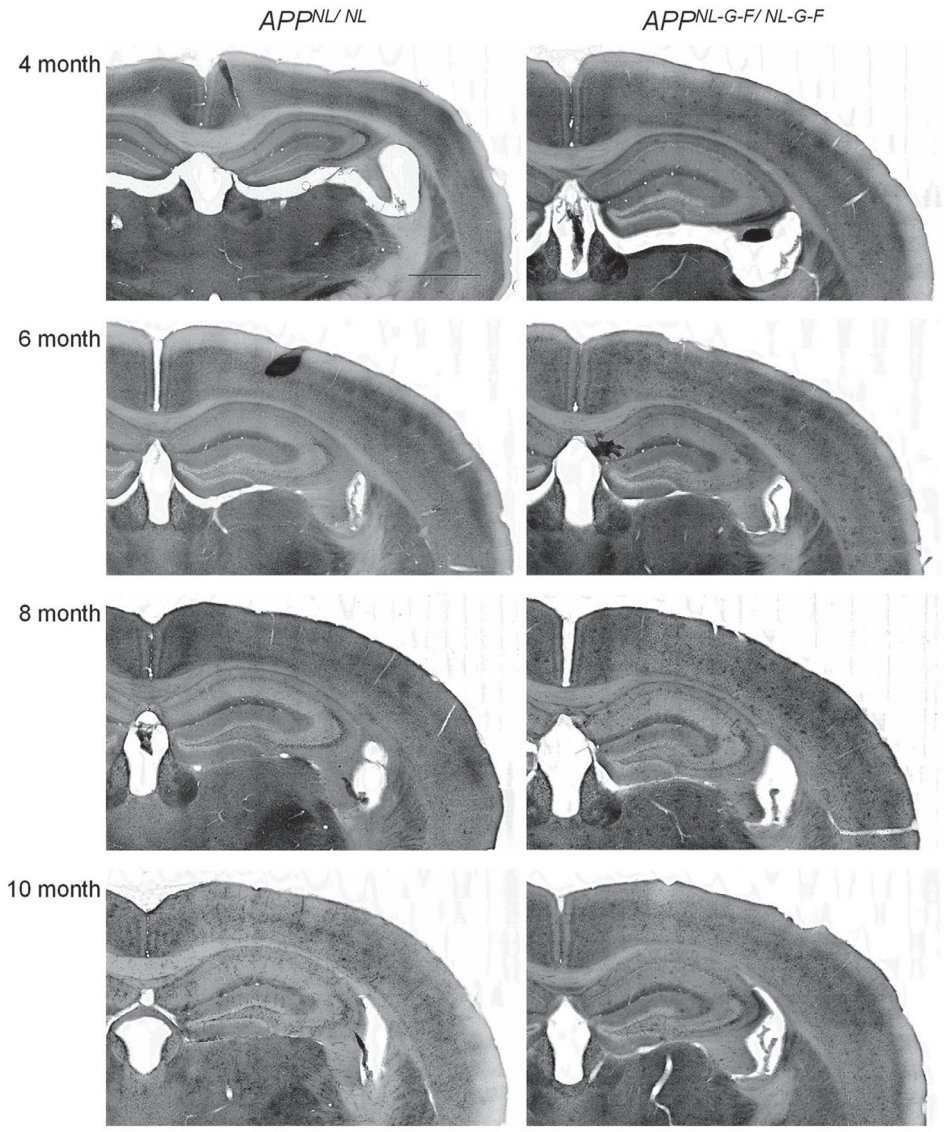


Fig. 3. Immunofluorescence Staining of CHOP in *App*$^{NL/NL}$ and *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ Mice Brains

Brain sections of 4- to 10-month-old mice were immunostained using mouse anti-CHOP antibody L63F7. All photos have the same magnification. Scale bars represent 1,000 μm.

the increase in Aβ that was observed with age, CHOP expression in *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ mice brains was also increased with age. Collectively, our immunofluorescence staining results suggested that CHOP expression was associated with an increase in age-related Aβ expression in the brains of *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in AD model mice.

Various *in vivo* and *in vitro* studies have suggested that targeting ER stress and ER stress-mediated apoptosis contributes to AD recovery.[18,19] Nevertheless, owing to species differences between humans and mice, existing AD mouse models, such as 3xTg or 5XFAD, are unable to fully simulate the pathologic and clinical features of human AD. The *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in AD mouse model was developed to more closely resemble human pathology. In this study, CHOP expression in *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ brains was confirmed using immunostaining, supporting the possibility that ER stress plays an important role in AD development due to Aβ accumulation. Besides CHOP, numerous other factors (ER stress marker proteins) are associated with ER stress, which form a series of cascades, and the detection of these marker proteins in brain sections should be undertaken in future studies. Our study findings strongly suggest that evaluation of the localization and expression variation of a variety of ER stress markers, including CHOP, in brain tissue sections, rather than analysis using brain total proteins, will enable clarification of the true role of ER stress in AD pathogenesis. Thus, the *App*$^{NL\text{-}G\text{-}F/NL\text{-}G\text{-}F}$ knock-in AD mouse model is useful for assessing the significance of ER stress in human AD pathogenesis, therapy, and prevention.

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Conflict of interest The authors declare no conflict of interest.

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