

## Alzheimer's disease pathology in Nasu-Hakola disease brains

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### Summary

Nasu-Hakola disease (NHD) is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts, caused by genetic mutations of either triggering receptor expressed on myeloid cells 2 (*TREM2*) or TYRO protein tyrosine kinase binding protein (*TYROBP*), alternatively named DNAX-activation protein 12 (*DAP12*), both of which are expressed on microglia in the brain and form the receptor-adaptor complex that chiefly recognizes anionic lipids. *TREM2* transmits the signals involved in microglial survival, proliferation, chemotaxis, and phagocytosis. A recent study indicated that a loss of *TREM2* function causes greater amounts of amyloid- $\beta$  ( $A\beta$ ) deposition in the hippocampus of a mouse model of Alzheimer's disease (AD) owing to a dysfunctional response of microglia to amyloid plaques, suggesting that *TREM2* facilitates  $A\beta$  clearance by microglia. *TREM2/DAP12*-mediated microglial response limits diffusion and toxicity of amyloid plaques by forming a protective barrier. However, the levels of  $A\beta$  deposition in postmortem brains of NHD, where the biological function of the *TREM2/DAP12* signaling pathway is completely lost, remain to be investigated. By immunohistochemistry, we studied the expression of  $A\beta$  and phosphorylated tau (p-tau) in the frontal cortex and the hippocampus of five NHD cases. Although we identified several small  $A\beta$ -immunoreactive spheroids, amyloid plaques were almost undetectable in NHD brains. We found a small number of p-tau-immunoreactive neurofibrillary tangle (NFT)-bearing neurons in NHD brains. Because AD pathology is less evident in NHD than the full-brown AD, it does not play an active role in the development of NHD.

**Keywords:** Alzheimer's disease, amyloid- $\beta$ , Nasu-Hakola disease, phosphorylated tau

### 1. Introduction

Nasu-Hakola disease (NHD), also designated polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOS; OMIM 221770), is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts, caused by genetic mutations of either triggering receptor expressed on myeloid cells 2 (*TREM2*) or TYRO protein tyrosine kinase binding protein (*TYROBP*), alternatively named DNAX-

activation protein 12 (*DAP12*), both of which are expressed on microglia in the brain (1). Clinically, the patients with NHD show recurrent bone fractures during the third decade of life, and a frontal lobe syndrome during the fourth decade of life, and progressive dementia and death until the fifth decade of life (2). Pathologically, the brains of NHD patients exhibit extensive demyelination designated leukoencephalopathy, astrogliosis, accumulation of axonal spheroids, and remarkable activation of microglia predominantly in the white matter of frontal and temporal lobes and the basal ganglia (3). *TREM2*, expressed exclusively on microglia in the brain, serves as a receptor for anionic lipids, lipoproteins and apolipoproteins (4,5). *TREM2* forms a complex with *DAP12*, a signaling adaptor having an immunoreceptor tyrosine-based activation motif (ITAM) capable of recruiting the protein tyrosine kinase Syk that transduces a variety of downstream signals (6). *TREM2* transmits microglial signals involved in survival,

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proliferation, chemotaxis, and phagocytosis (7).

Alzheimer's disease (AD) is characterized by the hallmark pathology comprised of widespread amyloid- $\beta$  ( $A\beta$ ) deposition, formation of neurofibrillary tangles (NFTs) composed of abnormally phosphorylated tau (p-tau), extensive neurodegeneration, and profound activation of microglia in the brain (8). Recent studies identified rare genetic variants of *TREM2*, such as R47H and R62H, closely associated with an increased risk for development of AD (9).

A recent study showed that *TREM2* deficiency generates greater amounts of  $A\beta$  deposition in the hippocampus of 5XFAD mice, a mouse model of AD, due to a dysfunctional response of microglia to amyloid plaques, suggesting that *TREM2* facilitates  $A\beta$  clearance by microglia (4). *TREM2* interacts with fibrillar  $A\beta$  decorated with anionic and zwitterionic lipids (4).  $A\beta$ -lipoprotein complexes are efficiently taken up by microglia, depending on *TREM2* (5). *TREM2* deficiency induces apoptosis of microglia and reduces recruitment of microglia around  $A\beta$  plaques in the brains of mouse models of AD (4). Furthermore, microglial processes enriched in *TREM2* tightly surround early amyloid fibrils and plaques, and promote their compaction and insulation (10). In *TREM2*- or *DAP12*-deficient mouse models of AD, microglia showed a markedly reduced ability to envelope amyloid deposits, leading to an increase in less compact and more diffuse plaques associated with greater neuritic damage (10). These observations suggest that *TREM2*/*DAP12*-mediated microglial response limits diffusion and toxicity of amyloid plaques by forming a protective barrier (10,11). However, at present, the levels of  $A\beta$  deposition in postmortem NHD brains, where the biological function of *TREM2*/*DAP12* signaling pathway is completely lost, remain unknown. A previous study reported a 48-year-old man of NHD with numerous senile plaques and neurofibrillary tangles throughout the cerebral cortex (12), suggesting that an impaired *TREM2*/*DAP12* signaling function facilitates  $A\beta$  accumulation in the human brain. In the present study by immunohistochemistry, we investigate the expression of  $A\beta$  and p-tau in NHD brains to clarify whether the Alzheimer's disease pathology is augmented in NHD.

## 2. Materials and Methods

### 2.1. Human brain tissues

The brain autopsies were performed at the National Center Hospital, National Center of Neurology and Psychiatry (NCNP), Japan and affiliated hospitals of Research Resource Network (RRN), Japan. The comprehensive examination by an established neuropathologist (YS) validated the pathological diagnosis. In all cases, written informed consent was

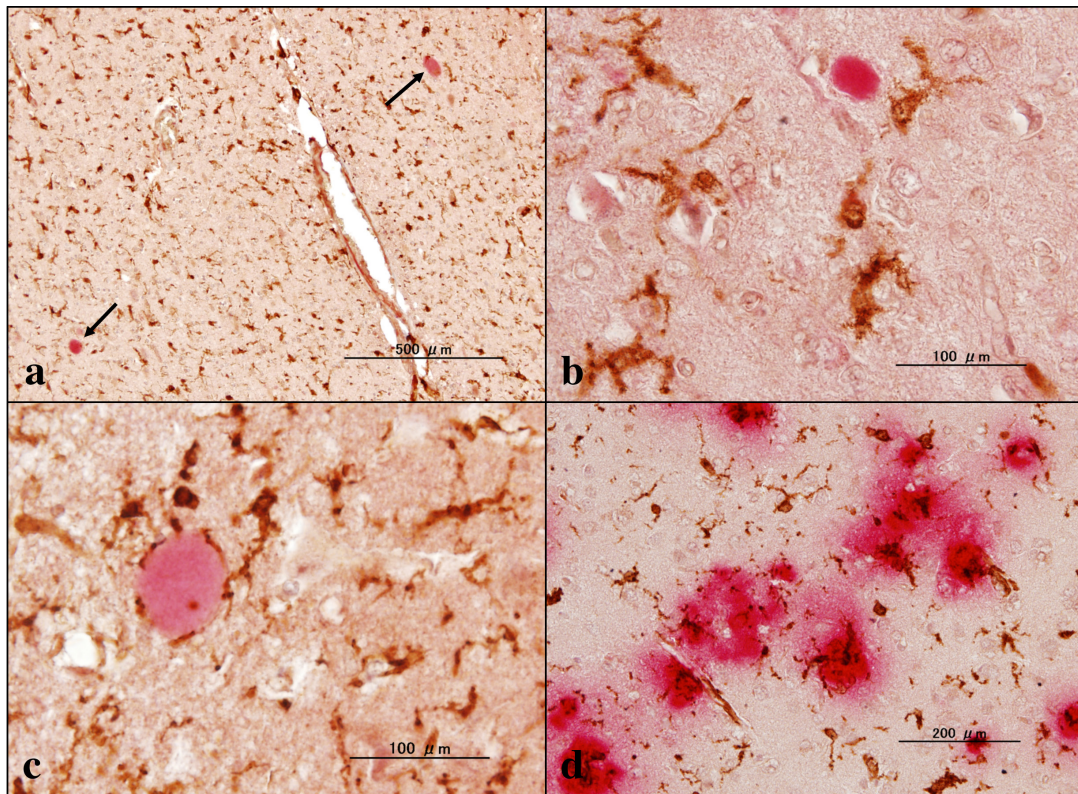
obtained. The Ethics Committee of the NCNP for the Human Brain Research and the Human Research Ethics Committee of the Meiji Pharmaceutical University (MPU) approved the present study.

For immunohistochemical studies, serial sections of the frontal cortex and the hippocampus were prepared from five AD patients, composed of a 68-year-old woman (AD1), a 68-year-old woman (AD3), a 56-year-old man (AD4), a 59-year-old man (AD5), and an 80-year-old man (AD8) and five NHD patients, composed of a 42-year-old man (NHD1), a 48-year-old woman (NHD2), a 44-year-old man (NHD3), a 32-year-old woman (NHD4), and a 38-year-old man (NHD5). The homozygous mutation of a single base deletion of 141G (c.141delG) in exon 3 of *DAP12* was identified in NHD1, NHD2, and NHD5, while the genetic analysis was not performed in NHD3 or NHD4. All AD cases were satisfied with the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria for diagnosis of definite AD (13). They were categorized into the stage C of amyloid deposition and the stage VI of neurofibrillary degeneration, following the Braak's staging (14).

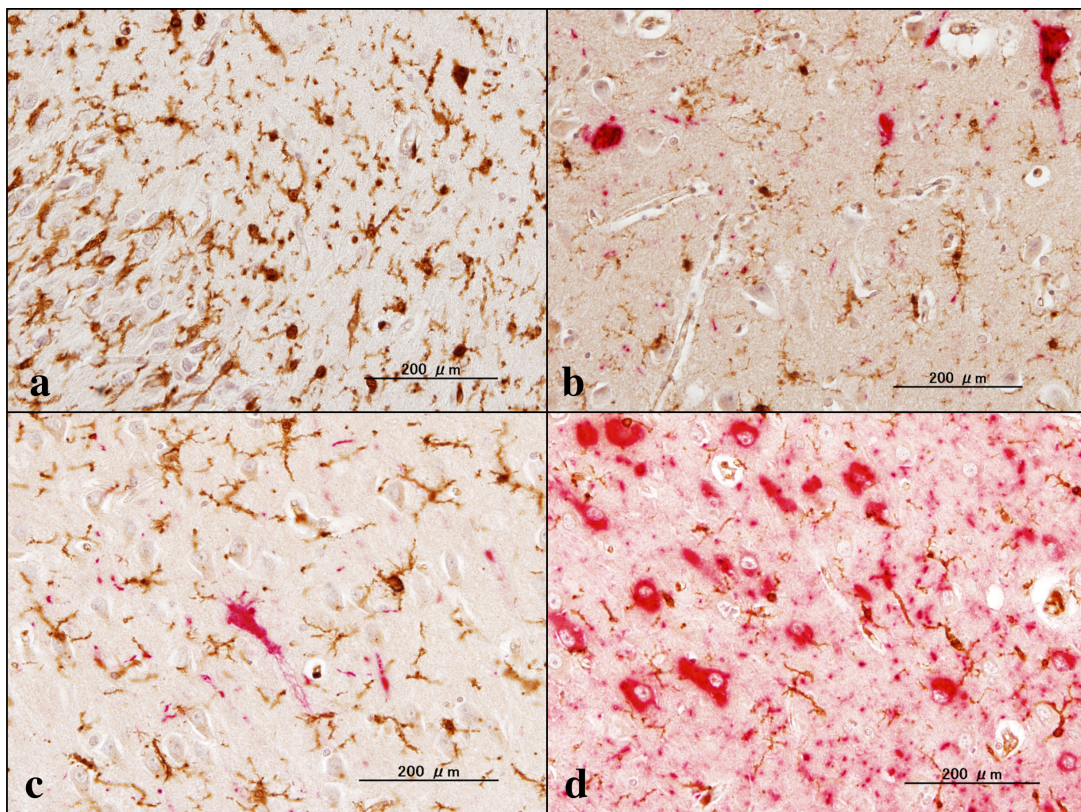
### 2.2. Immunohistochemistry

After deparaffination, tissue sections were heated in 10 mM citrate sodium buffer, pH 6.0 by autoclave at 110°C for 15 min in a temperature-controlled pressure chamber (Biocare Medical, Pacheco, CA, USA). Then, they were treated at room temperature (RT) for 5 min with formic acid before labeling with anti- $A\beta$  antibody. They were incubated with phosphate-buffered saline (PBS) containing 10% normal goat serum at RT for 15 min to block non-specific staining, followed by incubation in a moist chamber at 4°C overnight with mouse monoclonal anti- $A\beta$  peptide antibody at a concentration of 1  $\mu$ g/mL (12B2; Immunobiological Laboratories, Gunma, Japan) that reacts with  $A\beta$ 40,  $A\beta$ 42, and  $A\beta$ 43 or mouse monoclonal anti-phosphorylated tau (Ser202, Thr205) antibody at 0.025  $\mu$ g/mL (AT8; ThermoFisher Scientific, Waltham, MA, USA). After washing with PBS, tissue sections were incubated at RT for 30 min with alkaline phosphatase (AP)-conjugated secondary antibody (Nichirei, Tokyo, Japan), followed by exposure to Warp Red chromogen (Biocare Medical). For double immunolabeling, following heat treatment, tissue sections were treated with 3% hydrogen peroxide-containing water to block the endogenous peroxidase activity, and immunolabeled at 4°C overnight with rabbit polyclonal anti-Iba1 antibody at 0.5  $\mu$ g/mL (Wako Pure Chemical, Tokyo, Japan) for a marker specific for microglia. They were incubated at RT for 30 min with horseradish peroxidase (HRP)-conjugated secondary antibody (Nichirei), followed by exposure to diaminobenzidine tetrahydrochloride (DAB) substrate (Vector, Burlingame, CA, USA). The





**Figure 1. A $\beta$  immunoreactivity in NHD and AD brains.** (a) the hippocampus white matter of NHD (NHD4), A $\beta$  (red) and Iba1 (brown), spheroids (arrows), (b) the frontal lobe white matter of NHD (NHD5), A $\beta$  (red) and Iba1 (brown), spheroid, (c) the hippocampus white matter of NHD (NHD2), A $\beta$  (red) and Iba1 (brown), spheroid, and (d) the frontal cortex of AD (AD8), A $\beta$  (red) and Iba1 (brown), amyloid plaques.



**Figure 2. P-tau immunoreactivity in NHD and AD brains.** (a) the hippocampus of NHD (NHD5), p-tau (red) and Iba1 (brown), no NFT, (b) the hippocampus of NHD (NHD1), p-tau (red) and Iba1 (brown), several NFTs, (c) the hippocampus of NHD (NHD2), p-tau (red) and Iba1 (brown), one NFT, and (d) the hippocampus of AD (AD5), p-tau (red) and Iba1 (brown), numerous NFTs.



tissue sections were processed for a counterstain with hematoxylin. Negative controls underwent all the steps except for exposure to the primary antibody.

### 3. Results and Discussion

In mouse models of AD, the loss of function of TREM2 increases A $\beta$  plaque burden possibly through decreased phagocytic clearance of A $\beta$  by microglia (4). Loss of TREM2 function reduces the ability of microglia to engulf A $\beta$  (15). AD patients with the TREM2 variant of R47H showed fewer microglia surrounding plaques, increased numbers of filamentous non-compacted plaques, and more p-tau-positive neurites around plaques (10). In all NHD cases, we found several small A $\beta$ -immunoreactive deposits, and some of them might represent axonal spheroids located chiefly in the white matter of the frontal cortex and the hippocampus (Figure 1a-c). They were spherical in shape with smooth margins devoid of the core. Iba1+ microglia occasionally contacted these spheroids (Figure 1c). In support of these observations, a previous study showed the persistent accumulation of A $\beta$ 42 in axonal spheroids in a rat model of traumatic injury (16). In contrast, amyloid plaques, compact or diffuse, and amyloid angiopathy were almost undetectable in any cases. A $\beta$ -immunoreactive spheroids consisted of 4-22 spots/20 fields in the frontal cortex and 2-16 spots/20 fields in the hippocampus of NHD brains under microscopic examination at a magnification of 200  $\times$ . In contrast, the deposition of A $\beta$  was much extensive in AD brains (Figure 1d). The omission of the primary antibody did not show any positive reactions. In NHD brains except for NHD5, we identified a small cluster of NFT-bearing neurons labeled by anti-p-tau antibody located predominantly in the hippocampus (Figure 2a-c). NFT-bearing neurons consisted of 0-11 neurons/20 fields in the frontal cortex and 0-34 neurons/20 fields in the hippocampus of NHD brains under microscopic examination at a magnification of 200  $\times$ . We found a trend for the age-dependent increase in p-tau-immunoreactive NFT-bearing neurons. In contrast, numerous p-tau-immunoreactive neuronal deposits, composed of NFTs and dystrophic neurites, were observed in AD brains (Figure 2d). These results indicated that the loss of function of TREM2/DAP12 signaling pathway does not accelerate AD pathology in NHD brains.

The discrepancy between TREM2-deficient AD mice showing greater amounts of A $\beta$  deposition in the hippocampus (4) and NHD patients not exhibiting the acceleration of A $\beta$  deposition in the frontal cortex and the hippocampus is attributable to a difference in species, ages, and genetic backgrounds. Plaque accumulation is exacerbated at later ages in a TREM2 knockout AD mouse model (17). In AD patients, A $\beta$  plaques appear a decade or two before clinical

symptoms of AD (18). In the present study, NHD patients are 32- to 48-year-old. Therefore, they could be affected by the early AD pathology according to their ages. Importantly, by using florbetapir-amyloid-positron emission tomography (PET), a recent study demonstrated extensive A $\beta$  deposition in the grey matter of the inferior frontal and occipital lobes of a 39-year-old Italian NHD woman with a homozygous Q33X mutation in TREM2, suggesting the existence of overlapping pathogenic mechanisms between NHD and AD (19). In the present study, three cases of NHD showed the homozygous mutation of c.141delG in exon 3 of DAP12. A recent study showed that deficiency of DAP12 does not modify the number and size of A $\beta$  plaque deposition in the prefrontal cortex and the hippocampus of APP/PSEN1 mice, although DAP12 deficiency reduces plaque compaction, microglial clustering, and phagocytosis (20). Furthermore, phosphorylation of tau is attenuated in female APP/PSEN1 mice with loss of DAP12. In contrast, silencing of brain TREM2 exacerbates tau pathology in P301S tau transgenic mice, associated with neuroinflammation-induced overactivation of tau kinases, such as cyclin dependent kinase 5 (CDK5) and glycogen synthase kinase 3 beta (GSK3B) (21). Thus, the absence of either DAP12 or TREM2 produces apparently opposing effects on progression of AD pathology in mouse models of AD.

In summary, we identified no obvious A $\beta$  plaques and a small number of p-tau-immunoreactive NFT-bearing neurons in NHD brains. Because AD pathology is less evident in NHD than the full-brown AD, it does not play an active role in the development of NHD.

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