Porphyromonas gingivalis outer membrane vesicles in cerebral ventricles activate microglia in mice

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Conflict of interest disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval statement

Mice were housed under specific pathogen-free conditions according to the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions (the Ministry of Education, Culture, Sports, Science and Technology, Japan, 2006). All animal experiments were approved by the Ethics Committee of Animal Care and Experimentation of Tokushima University (approval number: T29-31).

Abbreviations

Pg, *Porphyromonas gingivalis*; AD, Alzheimer's disease; OMV, outer membrane vesicle; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; ATCC, American Type

Culture Collection; CSF, cerebrospinal fluid; BBB, blood brain barrier; BCSFB, blood-cerebrospinal fluid barrier

Running title

P. gingivalis OMVs induce neuroinflammation

Abstract

Objective: *Porphyromonas gingivalis* (*Pg*) is thought to be involved in the progression of Alzheimer's disease (AD). Whether *Pg* or its contents can reach the brain and directly affect neuropathology is, however, unknown. Here, we investigated whether outer membrane vesicles (OMVs) of *Pg* translocate to the brain and induce the pathogenic features of AD.

Material and Methods: *Pg* OMVs were injected into the abdominal cavity of mice for 12 weeks. *Pg* OMV translocation to the brain was detected by immunohistochemistry using an anti-gingipain antibody. Tau protein and microglial activation in the mouse brain were examined by western blotting and immunohistochemistry. The effect of gingipains on inflammation was assessed by real-time polymerase chain reaction using human microglial HMC3 cells.

Results: Gingipains were detected in the region around cerebral ventricles, choroid plexus, and ventricular ependymal cells in *Pg* OMV-administered mice. Tau and phosphorylated Tau protein increased and microglia were activated. *Pg* OMVs also increased the gene expression of proinflammatory cytokines in HMC3 cells in a gingipain-dependent manner.

Conclusion: *Pg* OMVs, including gingipains, can reach the cerebral ventricle and induce neuroinflammation by activating microglia. *Pg* OMVs may provide a better understanding of the implications of periodontal diseases in neurodegenerative conditions such as AD.

Keywords *Porphyromonas gingivalis*, outer membrane vesicle, gingipain, neuroinflammation, microglia, cerebral ventricle

1. Introduction

Porphyromonas gingivalis (*Pg*) is a gram-negative bacterium known to be a key pathogen in periodontal diseases (Genco & Borgnakke, 2013). *Pg* expresses various types of virulence factors, including the gingipain family proteins, arginine-specific gingipains (RgpA and RgpB) and lysine-specific gingipain (Kgp), that contribute to the progression of periodontal diseases by destroying periodontal tissues (Gabarrini, Grasso, van Winkelhoff, & van Dijl, 2020; O'Brien-Simpson, Pathirana, Walker, & Reynolds, 2009). Periodontal diseases have been implicated in the pathogenesis of Alzheimer's disease (AD) (Ingar Olsen & Singhrao, 2020). Gingipains have been detected in the brains of patients with AD, and small-molecule inhibitors of gingipains are known to rescue the neurodegeneration in the *Pg*-infected brain (Dominy et al., 2019). *Pg* lipopolysaccharide (LPS) has also been detected in the brains of AD patients (Poole, Singhrao, Kesavalu, Curtis, & Crean, 2013). In mouse models, *Pg* infection can exacerbate the features of AD with an increase in *Pg* LPS in the brain (Ishida et al., 2017).

AD is a neurodegenerative disease characterized by chronic inflammation of the brain (Akiyama et al., 2000). The microglia, which are immune effective cells in the brain, are activated by amyloid deposits and secrete a variety of pro-inflammatory cytokines, which contribute to the progression of AD (Mandrekar-Colucci & Landreth, 2010). *Pg* has been proposed to induce neuroinflammation by activating microglia in AD (I. Olsen, 2021). *Pg* LPS-induced periodontitis impairs recognition in rats through neuroinflammation by activating microglia (Yoshiyama et al., 2007). Gingipains have also been reported to be responsible for the migration and activation of microglia by *Pg* (Liu et al., 2017; Nonaka & Nakanishi, 2020). These observations suggest that *Pg* infection in the oral cavity may affect the distant brain tissue and increase the risk of AD. However, it is still debatable whether *Pg* or its contents such as LPS and gingipains can cross anatomical structures such as the blood-brain barrier (BBB), which inhibits the penetration of small molecules from the blood to the central nervous system and prevents neuropathology in the brain.

Similar to other gram-negative bacteria, *Pg* releases abundant outer membrane vesicles (OMVs) during its growth in various environments, including lipid culture and biofilms. Given their small size (50-70

nm in diameter) and adhesive and proteolytic properties, OMVs are thought to fuse with host cells and deliver their contents directly into the cytosol. Further, OMVs can spread more readily in the tissue than in bacterial cells (Kuehn & Kesty, 2005). *Pg* OMVs contain most of *Pg* virulence factors such as gingipains and LPS and are internalised into the host cell, which results in modulation of various responses. For instance, gingipains present on the surface of *Pg* OMVs increased vascular permeability both *in vitro* and *in vivo* via proteolytic cleavage of endothelial cell-cell adhesion (Farrugia, Stafford, & Murdoch, 2020). Recently, we also observed that *Pg* OMVs injected into the abdominal cavity could reach the liver and inhibit hepatic glycogenesis, resulting in high blood glucose levels in mice (Seyama et al., 2020). These observations prompted us to hypothesize that *Pg* OMVs could be an alternative mechanism by which *Pg* transmits virulence factors to distant organs in the body and eventually induce extraoral diseases.

In this study, we examined whether *Pg* OMVs translocate to the brain and increase the risk of AD. We isolated OMVs from the culture medium of *Pg* and intraperitoneally injected them into mice. The translocation of *Pg* OMVs to the brain tissue was detected by immunohistochemistry using an anti-gingipain antibody. We also examined the effect of *Pg* OMVs on tau protein expression and microglial activation, which are pathogenic features of AD, using mouse or human microglial HMC3 cells.

2. Materials and Methods

2.1. Bacterial culture and isolation of Pg OMVs

Porphyromonas gingivalis (*Pg*) ATCC 33277 was cultured in brain heart infusion medium (BD Bioscience, Franklin Lakes, NJ, USA) containing 0.5% yeast extract (BD Bioscience), 10 μg/mL hemin (Wako Chemicals, Osaka, Japan), and 1 μg/mL 2-methyl-1, 4-naphthoquinone (Tokyo Kasei, Tokyo, Japan) in an anaerobic jar at 37°C. The gingipain mutant of *Pg* ATCC 33277 (KDP136) was kindly provided by Dr. Koji Nakayama (Nagasaki University) and cultured in the aforementioned medium containing 25 μg/mL chloramphenicol, 10 μg/mL erythromycin, and 1 μg/mL tetracycline.

126 *Pg* OMVs were isolated using an established protocol (Eguchi et al., 2018; Ono et al., 2018) as described
127 in our previous report (Seyama et al., 2020). Briefly, the culture medium of *Pg* was centrifuged at 2800
128 ×*g* for 15 min at 4 °C. The supernatant was filtered with a 0.2 μm syringe filter and then concentrated
129 using Ultra-15 Centrifugal Filter Device for nominal molecular weight limit (NMWL) 100,000
130 (Amicon, Merck, Tokyo, Japan). The concentrate was mixed with Total Exosome Isolation Reagent
131 (Thermo Fisher Scientific, Tokyo, Japan) and incubated overnight at 4°C. The sample was centrifuged
132 at 10,000 ×*g* for 60 min at 4°C, and the *Pg* OMV fraction was obtained by eluting the pellet in 100 μL
133 phosphate-buffered saline PBS (-).
134
135 ***2.2. Animals***
136 BALB/cAJc1 mice (female, 40 weeks old) were purchased from Japan CLEA (Tokyo, Japan) and fed
137 a high-fat diet (Quick Fat, 15.3% fat, 424.5 kcal/100 g; Japan CLEA). Mice were housed under specific
138 pathogen-free conditions according to the Fundamental Guidelines for Proper Conduct of Animal
139 Experiments and Related Activities in Academic Research Institutions (the Ministry of Education,
140 Culture, Sports, Science and Technology, Japan, 2006). All animal experiments were approved by the
141 Ethics Committee of Animal Care and Experimentation of Tokushima University (approval number:
142 T29-31).
143
144 ***2.3. Immunohistochemistry***
145 *Pg* OMVs (5 μg of total protein) or PBS was intraperitoneally injected into mice for 12 weeks at a 3-
146 day interval. After the indicated time periods, mouse brain tissues were excised and fixed in 4%
147 paraformaldehyde-containing phosphate buffer solution (Nacalai Tesque) for 48 h at 4°C. The tissues
148 were then embedded in paraffin wax and sectioned at 4 μm thickness. Paraffin was removed from
149 sections using xylene and ethanol. The sections were heated in a 0.1 M buffer to retrieve antigens,
150 followed by incubation in 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min. After blocking with
151 4% bovine serum albumin (BSA) in PBS for 60 min, the sections were incubated with anti-Kgp/Rgp

antibody (1:800), Iba1 (1:500, GTX100042, Gene Tex), or normal rabbit IgG for 16 h at 4°C. The rabbit polyclonal antibody against Kgp/Rgp was kindly provided by Dr. Tomoko Kadowaki of Nagasaki University (Takii, Kadowaki, Baba, Tsukuba, & Yamamoto, 2005). Immunoreactive sites were identified using the SignalStain Boost Detection reagent (Cell Signaling Technology) and observed using a BZ-X800 microscope (Keyence).

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis

After 12 weeks of *Pg* OMV administration, the brains were excised and homogenized in a lysate buffer (1 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/mL leupeptin, 4 μg/mL aprotinin, and 50 mM sodium fluoride [NaF]). Ten micrograms of each sample were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated for 1 h at ambient temperature in 5% non-fat skim milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-Tween) and then incubated overnight at 4°C in a blocking solution containing specific antibodies. The primary antibodies used were phospho-Tau (Ser202, Thr205) (AT8) (1:1000, MN1020, Thermo Fisher Scientific), Tau (D1M9X) (1:1000, #46687, Cell Signaling Technology), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000, 60008-1, Proteintech). After washing with PBS-Tween, the membranes were incubated for 1 h at room temperature in a blocking solution containing horseradish peroxidase-conjugated secondary antibodies (dilution, 1:5000). The membranes were then washed, and the signals were detected using a western blot chemiluminescence horseradish peroxidase (HRP) substrate (Takara) according to the manufacturer's instructions.

The images of the bands obtained by western blotting were scanned and quantified using ImageJ version 1.47. Densitometry of Tau and phospho-Tau was normalized to that of GAPDH. The ratios of Tau/GAPDH and phospho-Tau/GAPDH are shown.

2.5. Cell culture

Human embryonic microglial clone HMC3 cells (ATCC: CRL-3304) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HMC3 cells were plated in plastic dishes at a density of 10×10^4 cells/ mL in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under a humidified atmosphere of 5% CO_2.

2.6. Real-time PCR

After reaching 70-80% confluence, HMC3 cells were treated with or without Pg OMVs at a concentration of 500 ng/mL for 3 h. Total RNA was isolated from HMC3 cells using ISOGEN (Nippon Gene), according to the manufacturer's protocol. The cDNA was synthesised using the Prime Script™ RT Reagent Kit (Takara Bio, Kyoto, Japan). Real-time PCR was performed using the 7300 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) with SYBR Premix Ex Taq™ (Takara Bio). The following primer sequences were used: human GAPDH (NM_002046.7) forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'; human tumor necrosis factor-alpha (TNF-α, NM_000594) forward, 5'-GACAAGCCTGTAGCCCATGTTGTA-3' and reverse, 5'-CAGCCTTGGCCCTTGAAGA-3'; human interleukin-6 (IL-6; NM_000600.5) forward, 5'-AAGCCAGAGCTGTGCAGATGAGTA-3' and reverse, 5'-TGTCCTGCAGCCACTGGTTC-3'.

2.7. Statistical analysis

Statistical analyses were performed using the Statcel2 software. The normal distribution of the data was first examined using the chi-square test. Variables that had a normal distribution were analyzed using the Student's *t*-test.

3. Results

3.1. Gingipains were detected around the cerebral ventricle in Pg OMV-administered mice

To examine the effects of *Pg* OMVs on neuropathology, *Pg* OMVs or PBS was administered into the abdominal cavities of mice for 12 weeks and the brain tissues were excised after the treatment. We first confirmed whether intraperitoneally injected-*Pg* OMVs could reach the brains of mice. As we have previously shown that *Pg* OMVs contain the gingipain variants Kgp and Rgp (Seyama et al., 2020), the translocation of *Pg* OMVs to the brain was assessed by immunohistochemistry using antibodies against Kgp/Rgp.

In the brains of PBS-treated mice, no gingipain-specific staining was observed in any region (Fig. 1a, b). In the brains of *Pg* OMV-administered mice, gingipains were detected in the extracellular spaces in regions near cerebral ventricles (Fig. 1c-e). The positive reaction of gingipains was visualized as a dot-shaped deposition in the extracellular space (Fig. 1e, open arrowheads). Staining with normal rabbit IgG instead of anti-Rgp/Kgp antibody failed to detect a positive signal (Fig. 1f), indicating the gingipain-specific staining. These findings suggest that *Pg* OMVs injected into the abdominal cavity were translocated with their cargo gingipains into the mouse brain.

3.2. Gingipains were detected in the choroid plexus and ventricular ependymal cells of Pg OMV-administered mice

Interestingly, gingipains were detected only in the region near the cerebral ventricle, but not in the cerebral cortex, of *Pg* OMV-administered mice. This observation prompted us to further dissect the pathway of *Pg* OMVs to the brain from the bloodstream. As small molecules in the systemic blood are known to leak from the choroid plexus to the cerebrospinal fluid (CSF) (Ghersi-Egea & Strazielle, 2001), we investigated the presence of gingipains in the choroid plexus and ependymal cells surrounding the cerebral ventricle. Immunohistochemistry results showed no positive staining of gingipains in the choroid plexus of PBS-treated mice (Fig. 2a, 2b). In *Pg* OMV-administered mice, the positive signal of gingipains was observed in the perinuclear region of the choroid plexus epithelial cells (Fig. 2d, 2e, closed arrowheads) and blood vessels within the choroid plexus (Fig. 2e, arrows). Consistent with the results in Figure 1, gingipains were also detected in the extracellular spaces of the

brain around the ventricle (Fig. 2e, open arrowheads). The brain sections from *Pg* OMV-treated mice showed negative staining when probed with normal rabbit IgG (Fig. 2f).

We next determined the presence of gingipains in ependymal cells, the ciliated cuboidal cells without basal lamina that line the surface of the cerebral ventricle. Similar to the choroid plexus epithelial cells, gingipains were localised in the perinuclear regions of ependymal cells from *Pg* OMV-administered mice (Fig. 2d, 2g, closed arrowheads) but not in those from PBS-treated mice (Fig. 2c). In extracellular spaces near ependymal cells, gingipains were visualised as dot-shaped deposits (Fig. 2g, open arrowheads). These positive reactions with gingipains were not observed in the brain probed with normal rabbit IgG (Fig. 2h). Altogether, these results suggest that the choroid plexus and ependymal cells in cerebral ventricles are likely to be the pathways of *Pg* OMVs to the brain.

3.3. Tau and phosphorylated-Tau protein levels increased in the brains of Pg OMV-administered mice

Tau is a microtubule-associated protein that regulates the microtubule assembly and stabilises neurons. As Tau aggregation and phosphorylation could be the key events in the development of AD (Perea, Bolós, & Avila, 2020), we examined whether *Pg* OMV administration modulates the status of Tau protein in the brain of mice. Brain tissues were excised from the mice injected with *Pg* OMVs or PBS for 12 weeks and prepared for western blotting. As shown in Figure 3A, the expression levels of Tau and phosphorylated-Tau (p-Tau) increased in the brains of *Pg* OMV-administered mice (Fig. 3A). The results of western blotting for total Tau and phosphorylated Tau proteins were quantified using ImageJ software and compared between the groups. The Tau /GAPDH ratio was significantly higher in the brains of *Pg* OMV-treated mice (2.426 ± 0.155, $p = 0.002$) than in the brains of PBS-treated mice (1.000 ± 0.434). The administration of Pg OMVs also increased the p-Tau /GAPDH ratio (1.881 ± 0.157, $p = 0.013$) compared to that in the PBS-treated group (1.000 ± 0.406).

The ratios of Tau/GAPDH and p-Tau/GAPDH in each group are presented for mice treated with PBS

(n = 4) or *Pg* OMV (n = 4) and analysed by the Student's *t*-test. Values are shown as the fold change in expression levels in the PBS group. * $p < 0.05$, ** $p < 0.01$ as compared with mice treated with PBS. Both total and phosphorylated Tau protein levels were significantly higher in the brains of *Pg* OMV-administered mice than in the brains of PBS-treated mice (Fig. 3B).

3.4. Microglia were activated in the brains of Pg OMV-administered mice

Microglia are activated under pathological conditions and play a complex role in AD progression (Mandrekar-Colucci & Landreth, 2010). Therefore, we examined whether *Pg* OMVs affected microglial activation. The activated microglia showed morphological changes characterized with an amoeboid cell body with short processes (Diz-Chaves, Pernía, Carrero, & Garcia-Segura, 2012). Therefore, we visualized microglia by staining with an antibody specific for the microglial marker Iba1 and observed their morphology in the brains of both groups of mice.

In PBS-treated mice, several Iba1-immunoreactive cells were observed near the cerebral ventricle (Fig. 4A, a, b). Most of these cells had a small cell body with longer cell processes (Fig. 4A, c). Iba1-immunoreactive cells were also detected around the cerebral ventricle in *Pg* OMV-administered mice (Fig. 4A, d, e). In these mice, the proportion of cells with amoeboid cell bodies was higher than that observed in control mice (Fig. 4A, f). We also examined *Pg* OMV effects on microglia in the hippocampus. Similar to the observation around the cerebral ventricle, *Pg* OMV administration increased the number of microglia with amoeboid cell bodies and the intensity of Iba1 staining (Fig. 4B, d-f) in the hippocampus; these types of cells were not observed in PBS-treated mice (Fig. 4B, a-c). These results suggest that *Pg* OMV administration induced the activation of microglia in the mouse brain.

3.5. Pg OMVs increased the expression of proinflammatory cytokine genes in a gingipain-dependent manner in HMC3 cells

Although our histological experiments (Figs 1, 2, and 4) suggested that *Pg* OMVs, including gingipains, could translocate to the brain where microglia are activated, whether gingipains are involved in the activation of microglia is yet unknown. As activated microglia produce cytokines to promote neuroinflammation and exacerbate neuropathology in AD (W. Y. Wang, Tan, Yu, & Tan, 2015), we examined the effects of *Pg* OMVs on the expression of pro-inflammatory cytokine genes in human microglial HMC3 cells.

OMVs were isolated from the culture medium of the KDP136 strain of *Pg*, which is Kgp/Rgp double-deficient. We previously confirmed that OMVs of the KDP136 strain lack both Kgp and Rgp gingipains (Seyama et al., 2020). HMC3 cells were incubated with OMVs derived from the wild-type *Pg* (*Pg* OMVs [WT]) or KDP136 (*Pg* OMVs [K136]) strain for 3 h, and the expression of genes was analyzed by real-time PCR. As shown in Figure 5, treatment with *Pg* OMVs (WT) significantly increased the expression of inflammatory genes such as *IL-6*, *TNF-α*, *IL-8*, and *IL-1β*. In contrast, *Pg* OMVs (K136) failed to upregulate the expression of these genes (Fig. 5). These results suggest that gingipains on *Pg* OMVs are responsible for the induction of pro-inflammatory cytokine expression in HMC3 cells.

4. Discussion

The presence of *Pg* OMVs in body fluids such as blood, saliva, and GCF of patients with periodontal diseases has not been investigated to date, whereas genomic DNA qPCR revealed that the relative levels of salivary *Pg* OMVs increased in the periodontitis group when compared to that in the healthy group (P. Han, Bartold, Salomon, & Ivanovski, 2021). Therefore, we injected *Pg* OMVs (5 mg of protein) into mice, which affected glucose metabolism in a mouse model as reported in our previous study (Seyama et al., 2020).

Pg OMV has been considered as an alternative factor that causes neurodegenerative diseases in the brain, such as AD, because of its small size and rich content of virulence factors (Nara, Sindelar, Penn, Potempa, & Griffin, 2021; Singhrao & Olsen, 2018). However, there is no direct evidence that *Pg* OMV can access the brain and is implicated in AD pathogenesis. In this study, we first examined whether *Pg*

OMVs translocate to the mouse brain. As bacterial OMVs lack known unique biological markers, we attempted to track *Pg* OMVs by detecting their cargo protein gingipains. In cultured cells, but not in mouse models, *Pg* virulence factors such as gingipains and fimbriae are often used to detect the localization of *Pg* OMVs. For instance, Furuta et al. observed that *Pg* OMVs probed with antibodies against native fimbriae could invade HeLa and human gingival epithelial cells via a lipid-raft endocytic pathway (Furuta et al., 2009). Cy5-labelled *Pg* OMVs overlapped with immunostaining signals with the antibody against gingipain, indicating that gingipain could be used to detect the localisation of *Pg* OMVs. Consistent with these reports, positive staining for gingipain was observed in the brains of *Pg* OMV-administered mice (Figs 1 and 2), suggesting that *Pg* OMVs injected into the abdominal cavity may be translocated to the brain via the bloodstream in the body; however, the presence of gingipains or *Pg* OMVs in the blood serum was not determined.

The mechanisms underlying the translocation of *Pg* OMVs to the brain remain unclear. Both *in vitro* and *in vivo* experiments have revealed that *Pg* OMVs increase vascular permeability by disrupting tight junction proteins of endothelial cells in the systemic and cerebral vasculature in a gingipain-dependent manner (Farrugia, Stafford, & Murdoch, 2020; Farrugia, Stafford, Potempa, et al., 2020; Nonaka, Kadowaki, & Nakanishi, 2022; Pritchard et al., 2022). These properties are thought to enable *Pg* OMVs to pass through the BBB in the CNS; however, there have been no reports of direct visualisation of *Pg* OMVs in the brain. Recently, OMVs from another periodontal bacterium, *Aggregatibacter actinomycetemcomitans (Aa*), were tracked to the brain using the clearing technique and two-dimensional light sheet fluorescence microscopy analysis (E. C. Han et al., 2019). The lipophilic tracer DiD-labeled *Aa* OMVs were detected in the cortical extracellular space around the microvessels 24 h after intracardiac injection. These *Aa* OMVs were also co-localized with RNAs inside OMVs in the cortex region, indicating that *Aa* OMVs can pass through the BBB and are present in the cortex of mouse brains. However, in contrast to *Aa* OMVs, we detected positive staining of gingipains only in/near the region of the lateral ventricle, but not in other parts of the brain such as the cortex (Figs 1 and 2).

Although the BBB has been mainly investigated as the pathway by which bacterial OMVs enter the brain, small molecules in the systemic blood are known to leak from the choroid plexus into the CSF (Ghersi-Egea & Strazielle, 2001). This barrier between the choroid plexus and CSF is called the blood-cerebrospinal fluid barrier (BCSFB). The choroid plexus consists of blood vessels embedded in the stroma that are enclosed by epithelial cells. As the vascular endothelial cells herein are fenestrated and the capillaries are thin-walled, unlike those in the BBB, solutes in the blood can easily leak into the stroma of the choroid plexus (Johanson et al., 2017). The epithelium enclosed within the choroid plexus has low electrical resistance and is much more permeable than the BBB (Nguyan Hoang, 2019). Therefore, solutes in the stroma diffuse into the CSF. The ependymal cells lining the ventricle have incomplete tight junctions, which transport the solutes in the CSF to the extracellular spaces of the brain (Lippoldt et al., 2000; Oliver et al., 2013). These observations indicate that BCSFB has more permeable features than BBB; therefore, it was recently proposed as an alternative route for the uptake of *Pg* OMVs by the brain (Nara et al., 2021).

Indeed, we detected gingipains in the choroid plexus and ventricular ependymal cells, in addition to the extracellular spaces around the ventricle (Figs 1 and 2), suggesting that *Pg* OMVs possibly leaked from the blood to the brain via BCSFB. Notably, the gingipain-detected region was located around the inferior horn of the lateral ventricle. This part of the lateral ventricle contains abundant choroid plexus and is located downward following the outer surface of the hippocampus. These anatomical structures seem to allow *Pg* OMVs to reach the lateral ventricle, suggestive of its involvement in the pathogenesis of AD through its effect on the hippocampus. The report showing that *Pg* DNA was detected in the CSF of patients diagnosed with probable AD seems to be consistent with this concept (Dominy et al., 2019). Further investigations are warranted to track the pathway by which *Pg* OMVs enter the brain using, for example, an *in vivo* imaging system.

In this study, we also found that the microglia around the lateral ventricle changed their morphology to cells with amoeboid cell bodies in *Pg* OMV-injected mice (Fig. 4). These changes are consistent with the characteristics of activated microglia, as reported by Diz-Chaves et al, such as type IV microglia

(cells with large somas and retracted, thicker processes) and type V microglia (cells with ameboid cell bodies, numerous short processes, and intense Iba1 immunostaining) (Diz-Chaves et al., 2012). Activated microglia in the CNS secrete proinflammatory cytokines and contribute to neuroinflammation (Tsuda, Inoue, & Salter, 2005). Indeed, we found that *Pg* OMVs increased the levels of pro-inflammatory cytokines such as IL-6, TNF-α, IL-8, and IL-1β in a gingipain-dependent manner in HMC3 cells (Fig. 5). Further experiments are needed to determine the expression levels of these cytokines in gingipain-dependent brain tissue. Moreover, Tau protein was hyperphosphorylated in the brains of *Pg* OMV-administered mice (Fig. 3). Both microglial activation and Tau hyperphosphorylation have been shown to be related to the progression of AD (Perea et al., 2020). The Tau protein is abnormally hyperphosphorylated and aggregates into neurofibrillary tangles (NFTs) in the brains of patients with AD (J. Z. Wang, Xia, Grundke-Iqbal, & Iqbal, 2013). This hyperphosphorylation of Tau is thought to impair the binding affinity of Tau to microtubules in neurons, resulting in loss of neuronal integrity (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011). Based on these observations, it is possible that gingipains on *Pg* OMVs could induce neuroinflammation by activating microglia; however, the direct effects of gingipains on microglia in the mouse brain were not elucidated in the present study.

5. Conclusion

In summary, we show that *Pg* OMVs administered into the abdominal cavity were translocated to the region around the lateral ventricle in the brain, as demonstrated by immunostaining for gingipains. *Pg* OMVs were likely to reach around the lateral ventricle via the BCSFB because gingipains were also detected in the choroid plexus and ventricular ependymal cells. In the *Pg* OMV-treated mouse brain, both microglial activation and Tau hyperphosphorylation were observed. *Pg* OMVs also increased the gene expression of proinflammatory cytokines in HMC-3 cells in a gingipain-dependent manner. Our results suggest that *Pg* OMVs can reach the brain via the BCSFB and induce neuroinflammation by

activating microglia. These properties of *Pg* OMVs may provide a better understanding of the implications of periodontal diseases in neurodegenerative conditions such as AD.

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Figure Legends

Figure 1: Gingipains were detected around the cerebral ventricle in *Pg* OMV-administered mice

The mice were administered PBS (a, b) or *Pg* OMVs (c-f) for 12 weeks, and the brain tissue sections were stained with an anti-gingipain antibody (a-e) or normal rabbit IgG (f). The regions of the red boxes (a, c) are shown at higher magnification (b, d). A higher magnification of the region of the black box in (d) is shown in (e) and (f). Open arrowheads indicate specific anti-gingipain antibody staining (d and e). Scale bars indicate 200 μm (a, c), 100 μm (b, d), or 20 μm (e, f). Abbreviations: Hp, hippocampus; V, cerebral ventricle; Cp, choroid plexus.

Figure 2: Gingipains were detected in the choroid plexus and ventricular ependymal cells of *Pg* OMV-administered mice

Immunohistochemical detection of gingipains in the choroid plexus (b, e, f) and ventricular ependymal cells (c, g, h) of brain tissues from mice administered PBS (a–c) or *Pg* OMVs (d–h) for 12 weeks. The regions indicated by red boxes in (a) and (d) are shown at higher magnifications in (b, d) and (e, g), respectively.

Specific gingipain staining in the perinuclear region of the choroid plexus epithelial cells (e, closed arrowheads), blood vasculature within the choroid plexus (e, arrows), and extracellular spaces (e, opened arrowheads). Detection of gingipains in the perinuclear regions of ependymal cells (g, closed arrowheads) and extracellular spaces (g, opened arrowheads). The scale bars represent 200 μm (a, d), or 20 μm (b, c, e, f, g, h). Hp, hippocampus; V, cerebral ventricle; Cp, choroid plexus.

Figure 3: Tau and phosphorylated-Tau protein levels increased in the brains from *Pg* OMV-administered mice

Brains were excised from the mice administered PBS or *Pg* OMVs for 12 weeks. (A) Lysates of the brain tissue were analyzed by western blotting using anti-phospho-Tau (p-Tau), anti-Tau, and GAPDH. (B) Densitometry of Tau and phosphorylated-Tau (p-Tau) was normalized to that of GAPDH. The ratios

of Tau/GAPDH and p-Tau/GAPDH in each group are presented for mice treated with PBS (n = 4) or *Pg* OMV (n = 4) and analyzed by the Student's *t*-test. Values are shown as the fold change in expression levels in the PBS group. * $p < 0.05$, ** $p < 0.01$ as compared with mice treated with PBS.

Figure 4: Microglia were activated in the brains from *Pg* OMVs-administered mice

The mice were administered PBS (a-c) or *Pg* OMVs (d-f) for 12 weeks, and the brain tissue sections were stained with anti-Iba1 antibody. Iba1 staining in the region around the cerebral ventricle (A) and hippocampus (B). The region of the red boxes (a, d) is shown at a higher magnification (b, e). Higher magnifications of b and e are shown in c and f, respectively. Open arrowheads indicate activated microglia. Scale bars indicate 200 μm (a, d), 50 μm (b, e), and 20 μm (c, f).). The scale bars indicate 20 μm. "V" means cerebral ventricle.

Figure 5: *Pg* OMVs increased the expression of proinflammatory cytokine genes in a gingipain-dependent manner in HMC3 cells

HMC3 cells were treated with 500 ng/mL *Pg* OMVs for 3 h. The mRNA expression in HMC3 cells treated with PBS (n = 4), *Pg* OMVs (WT) (n = 4), or *Pg* OMVs (K136) (n = 4) was analyzed using real-time PCR. Values are shown as the fold change in expression levels in the PBS group. Student's *t*-test was used for statistical analysis. * $p < 0.05$, ** $p < 0.01$ compared each group.

Figure 1. Gingipains were detected around the cerebral ventricle in *Pg* OMV-administered mice

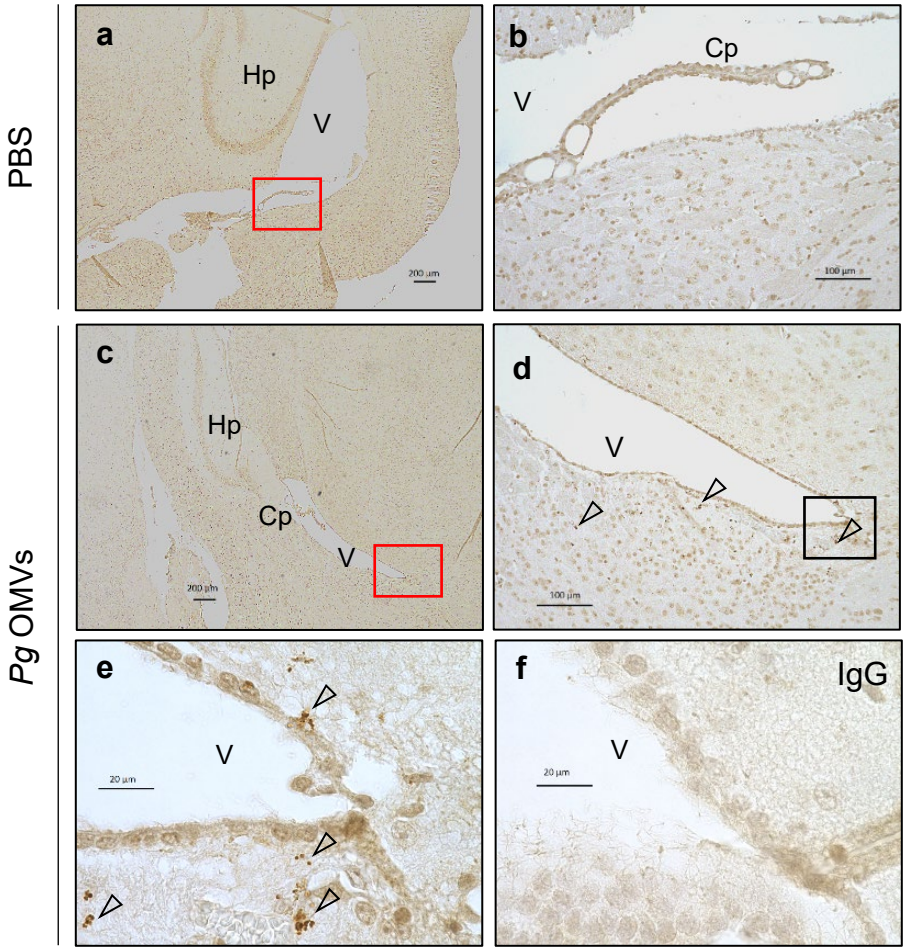


Figure 2. Gingipains were detected in the choroid plexus and ventricular ependymal cells of *Pg* OMV-administered mice

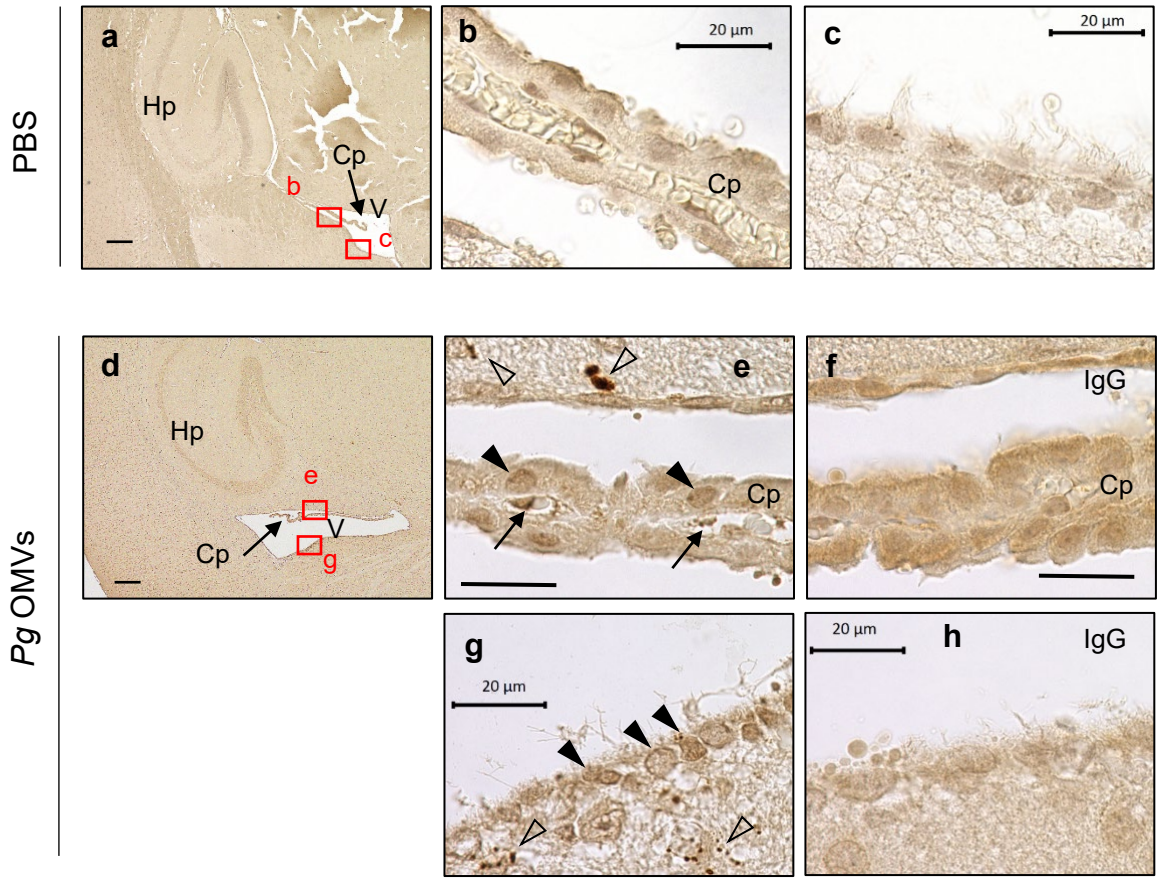
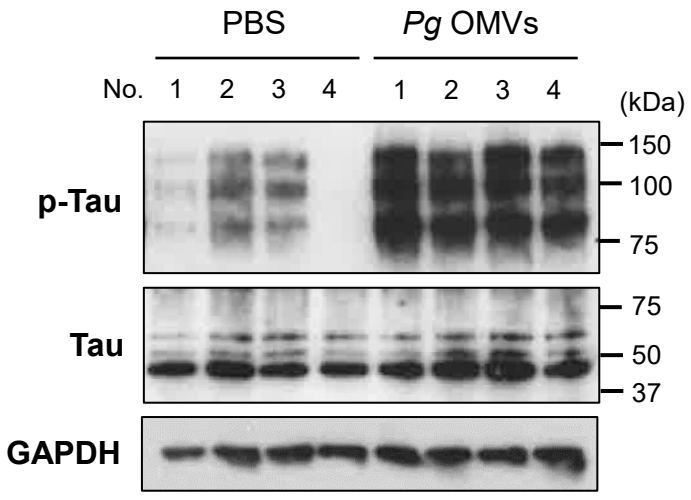


Figure 3. Tau and phosphorylated-Tau protein levels increased in the brains from *Pg* OMV-administered mice

A



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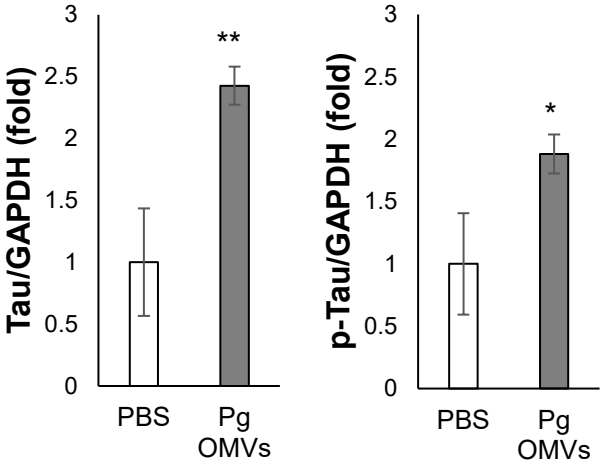
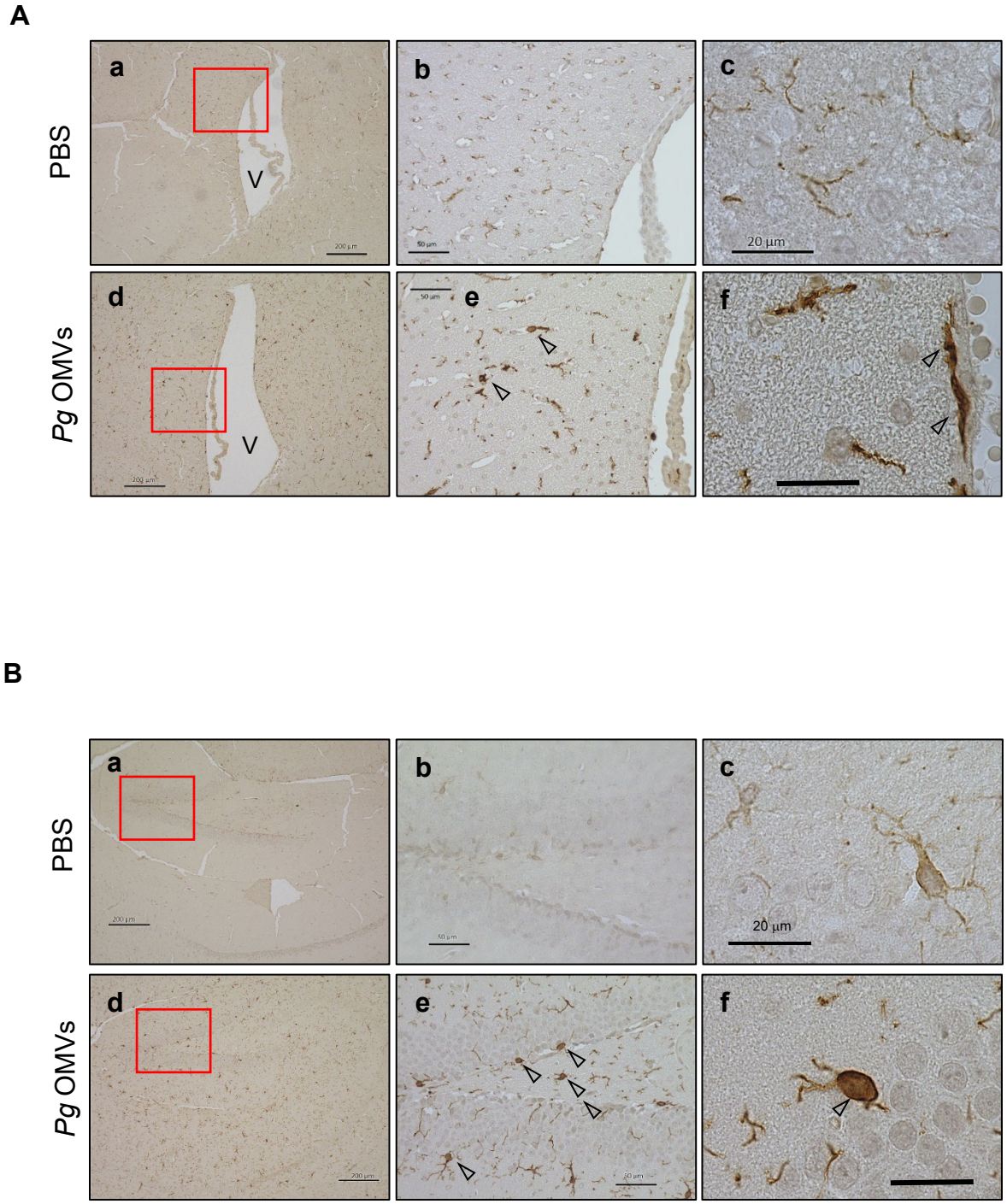


Figure 4. Microglia were activated in the brains from *Pg* OMVs-administered mice



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