

Gingipain Causes Tau Tangle Formation in Alzheimer's Disease Brains via Regulation of TREM-1

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Keywords: STREM-1, Gingipain, Tau, Alzheimer's Disease.

Abstract: Tau phosphorylation is widely believed as an indicator of Alzheimer's Disease (AD). Recent evidence suggests that the periodontal pathogen, *Porphyromonas gingivalis*, is associated with AD development by increasing the level of tau protein in the brain. Gingipain, the major virulence factor of the bacteria, has been described to be involved in the cleavage of the Triggering Receptor Expressed on Myeloid cells 1 (TREM-1). The soluble TREM-1 (sTREM-1) generated was shown to result in the activation of tau phosphorylation. This work aims to uncover the underlying mechanism by which *P. gingivalis* causes AD by studying the relationship between the level of gingipain, TREM-1 and tau in the brain of mice infected with *P. gingivalis*. Through this study, a novel mechanism of AD formation may be proposed and can be exploited to generate therapies against the disease.

1 INTRODUCTION

Alzheimer's disease (AD) is one of the leading causes of dementia worldwide. The widely accepted cause of AD is the loss of neurons caused by the formation of neurofibrillary tangles from tau phosphorylation (Niikura, Tajima, Kita 2006, Ryder 2020). However, the mechanism behind this process is not clear, and recent studies have shown that the gram-negative bacteria *Porphyromonas gingivalis* responsible for periodontal infections has been detected in the brain of AD patients, which suggest a role of the bacteria in the pathogenesis of the disease (Dominy et al 2019).

The major virulence factor produced by the bacteria is a family of conserved proteases called gingipain n (Dominy et al 2019). This family of protease consists of lysine-gingipain (Kgp), arginine-gingipain A (RgpA), and arginine-gingipain B (RgpB), which were found in the brain of 90% of the AD patients (Haditsch 2020). These proteases are localized to the hippocampus and result in the secretion of pro-inflammatory cytokines that cause damage to the brain (Ilievski et al 2018).

Experiments using human polymorphonuclear neutrophils have demonstrated a role of Rgp in the shredding of the triggering receptor expressed on

myeloid cells 1 (TREM1), which level is found to be higher in AD patients (Sao et al 2018). TREM1 enhances cellular response by the recruitment of more inflammatory cells (Bouchon, Dietrich, Colonna 2000). A study using human plasma demonstrated a positive relationship between the level of sTREM1 and tau protein in AD patients, thus confirming the role of sTREM1 in AD development (Jiang et al 2019).

The specific mechanism by which *P. gingivalis* induce AD has not been elucidated, and the connection between gingipain caused TREM-1 shredding and tau protein phosphorylation in AD patients has not been made. Here I proposed a model to investigate whether *P. gingivalis* can pass into the brain from oral infection by assessing the level of gingipain in the brain of mice. Then, the level of sTREM-1 in the brain will be determined to confirm the effect of *P. gingivalis* on TREM-1 shredding (Figure 1). To investigate the contribution of sTREM-1 in the formation of phosphorylated tau (phospho-tau), Trem-1 deficient mice will be used and compared with wild type mice for the level of phospho-tau. Lastly, as Rgp is shown to be responsible for TREM-1 shredding, the effect of a Rgp inhibitor will be investigated to prevent mice infected with *P. gingivalis* from AD.

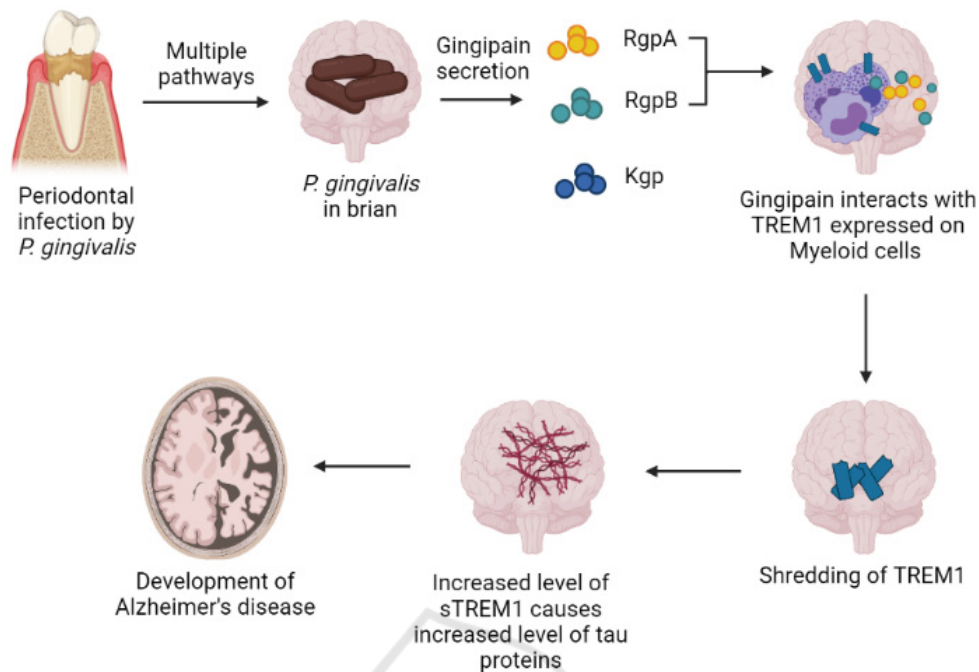


Figure 1: Proposed model for the development of Alzheimer's disease induced by *P. gingivalis* (Created with BioRender.com). *P. gingivalis* present at periodontal infections enters the brain via an unknown route, but several possible mechanisms have been proposed (Dominy et al 2019). Once inside the brain, gingipains are secreted and cleaves the TREM1 receptor present on myeloid cells into sTREM1, which then circulates in CSF and amplifies inflammatory responses. sTREM1 also cause rise in tau concentration and increased phosphorylation, which results in neuronal loss that contribute to AD development.

2 MATERIALS AND METHODS

2.1 Growth of *P. Gingivalis*

P. gingivalis (W83, ATCC® BAA308™) will be grown on tryptic soy agar plates under anaerobic condition in an anerobic gas chamber at 37°C for 3 days (ATCC® Medium 2722). *P. gingivalis* will then be harvested from the agar by scrapping and suspended in PBS + 2% methylcellulose at a concentration of 1×10^{10} cells/ml after washing with phosphate-buffered saline (PBS). Cell concentration will be determined by using a counting chamber under a microscope.

2.2 Animal

Female 5XFAD mice (#34840) from the Jackson Laboratory will be purchased and used for infection with *P. gingivalis*. Mice needs to be maintained under specific pathogen free conditions and fed with LabDiet® 5K52 formulation (6% fat) with tap water. The control and bacterially infected mice will be

maintained in separate cages and kept under a regular 12h dark/light cycle with a temperature and humidity of 22°C and 60, respectively.

2.3 Generation of Trem-1 Knockout Mice

The generation of Trem-1 knockout mice will follow the procedure outlined by Weber et al, 2014 (Weber et al 2014). Briefly, a vector based on KS loxP ftr Neo BS cloning vector need to be constructed in order to delete exon 2 of the Trem-1 gene. Deletion blocks TREM-1 activity as exon2 encodes the extracellular region and the ligand-binding site. Additional restriction sites (AseI and AvaI), and the positive selection markers, PuroR and Neomycin, is added for the selection. A Tk counterselection cassette is also included. The vector needs to be electroporated into mice embryonic stem cells using a BAC plasmid. Further mating of the genetically modified mice with mice carrying Cre recombinase is required to obtain Trem-1 knockout mice.

2.4 *P. gingivalis* Oral Infection on Mice

Forty-three weeks old 5XFAD female mice were used for oral infection with *P. gingivalis*. The mice will be anaesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) by intraperitoneal injection. The eyes will be lubricated with ophthalmic ointment to prevent drying. Anaesthetized mice will then be tied around the upper maxillary left and right second molar with silk ligature (FS 5080, Dolphin Sutures). 100ml of the bacterial solution will be applied to the buccal surface of the maxillae of the mice in infection groups. This procedure needs to be repeated every other day for six weeks. 100ml of PBS + 2% methylcellulose will be applied to the control groups on the same days.

Figure 2.

2.5 Effect of *P. gingivalis* Infection on the Level of Gingipain in Brain

To study the level of gingipain in the brain of infected mice, 20 infected and 20 controls of 43 weeks old female 5XFAD mice were used. Mice brains need to be prepared as outlined by Clark et al, 2011 (Clark et al 2011). Briefly, brains extracted were sliced using a cryostat into 40 μ m thick sections and fixed onto slides. Then, immunohistochemistry will be performed following the procedure described by Dominy et al, 2019, where the Rgp antibody 18E6 (University of Georgia) needs to be added to the samples and the result will be visualized using UltraView Universal DAB Detection System (Ventana Medical Systems) (Dominy et al 2019).

2.6 Effect of *P. gingivalis* Infection on the Level of TREM-1 in Brain

Immune cells will be isolated from the brain of 20 infected and 20 control mice to determine TREM-1 levels. The cells will undergo flow cytometry using the procedure stated by Liu et al, 2019 (Liu et al 2019). Briefly, cells extracted will be suspended in Hank's balanced salt solution (ThermoFisher), and TREM-1 antibodies (R&D, clone 174031) will be added. Flowjo (Tree Star Inc.) will then be used to analyze the results.

2.7 Effect of *P. gingivalis* Infection on the Level of Strem-1 in Brain

To investigate the level of sTREM-1 in mice brains, cerebrospinal fluid (CSF) from 20 infected and 20 control 43 weeks old female 5XFAD mice were used.

CSF will be extracted into polypropylene tubes using the method proposed by Sakic, 2019 (Šakić 2019). Then, the samples were analyzed using the TREM1 mouse ELISA kit (EMTREM1, ThermoFisher) following the protocol.

2.8 Effect of TREM-1 Knockout on the Level of Total/Phospho-Tau

The level of tau was investigated in 20 infected TREM-1(+/+) and 20 infected TREM-1(-/-) 5XFAD mice. Mice will be anaesthetized by intraperitoneal injection with 4% chloral hydrate (10 mL/kg), and the level of total tau protein present in the brain of the mice can be determined using the Tau (total) mouse ELISA kit (KMB7011, ThermoFisher) following the protocol using brain homogenate. The level of phospho-tau protein present can be determined using the Tau (Phospho) [pS199] Mouse ELISA kit (KMB7041, ThermoFisher) following the protocol using brain homogenate. Sample obtained were analyzed using a spectrophotometer, and the absorbance is read at 450nm to determine the concentration.

2.9 Effect of Gingipain Inhibitor on the Level of Strem-1 and Phospho-Tau

To test the effect of gingipain inhibitor in treating AD, 40 43 weeks old female 5XFAD mice were infected with *P. gingivalis*. Twenty of them received Rgp inhibitor (A18522, Adooq Bioscience) in DMSO every day starting from week two by intravenous injection (10mg/kg). Control mice received only DMSO. Determine the level of sTREM-1 and phospho-tau in the brain of the mice as above.

2.10 Data Analysis

All statistical analysis of the data obtained will be performed using RStudio version 1.2.5042. One-way analysis of variance (ANOVA) will be performed to assess whether the difference between the sample means of the groups were significant. A Bonferroni test will then be performed to reduce false-positive results. Data will be considered significant at $P < 0.05$.

3 CONCLUSION

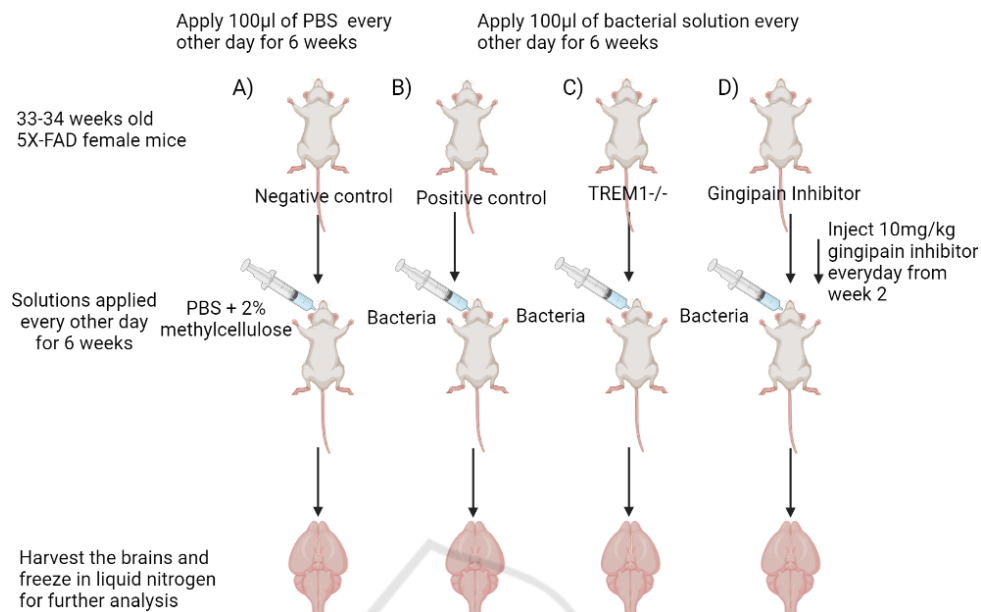


Figure 2: Experimental setup for sample collection of different groups (Created with BioRender.com). A) Negative control group, receiving only the solvent used to suspend the *P. gingivalis* cells. Act as a reference for the level of gingipain, TREM1 and tau protein in brain. B) Positive control, mice are infected with *P. gingivalis* to confirm that the infection will lead to an increase in TREM1 and tau proteins in brain. C) Mice with TREM1 knockout, to investigate whether TREM1 influences the level of tau protein in the brain. D) Mice treated with gingipain inhibitor, to investigate whether the effect is due to the presence of gingipain or not and also act as a possible treatment method for the infection.

This set of experiments could propose a new mechanism by which AD can be induced. Suppose the result meets the hypothesis: the bacterially infected mice have a higher level of gingipain, sTREM-1 and phosphorylated tau in the brain than control. In that case, it can be concluded that orally infected *P. gingivalis* is able to migrate into the brain and secretes gingipain that cleaves TREM-1 from cell surfaces. The increase in the level of sTREM-1 triggers the accumulation and phosphorylation of tau, which is a sign of AD. Suppose the inhibitor-treated mice demonstrated a lower level of sTREM-1 and phosphor-tau in the brain than positive control mice. It could be concluded that Rgp is responsible for the disease, and inhibition of this virulence factor could prevent AD development. However, other adverse effects of the replication bacteria inside the brain have not been determined and may contribute to the neurodegeneration seen in AD patients, thus requiring further characterization. This study only investigates the effect of Rgp, and the role of Kgp in the pathogenesis of the bacteria would need to be

determined to understand the complete mechanism by which *P. gingivalis* causes AD.

Although this set of experiments did not investigate the route by which *P. gingivalis* passes into the brain, but many models have been proposed and the mechanism of entry may contribute to the development of AD symptoms. For example, *P. gingivalis* may gain entry into the brain via direct damage to the endothelial cells of the blood-brain barrier (BBB) via its gingipains. As an earlier study has demonstrated the ability of gingipains secreted by the bacteria to induce apoptosis in endothelial cells (Sheets, Potempa, Travis, Casiano, Fletcher 2005). The damage to BBB are often seen as an early marker for the development of AD, and contributes to neurodegeneration that led to dementia. Thus, both the effect of the damage of BBB and cleavage of TREM1 need to be considered to fully characterize *P. gingivalis* infection on causing AD.

Overall, the experiments provided a pathway to investigate a novel cause of AD and presented a possible treatment method.

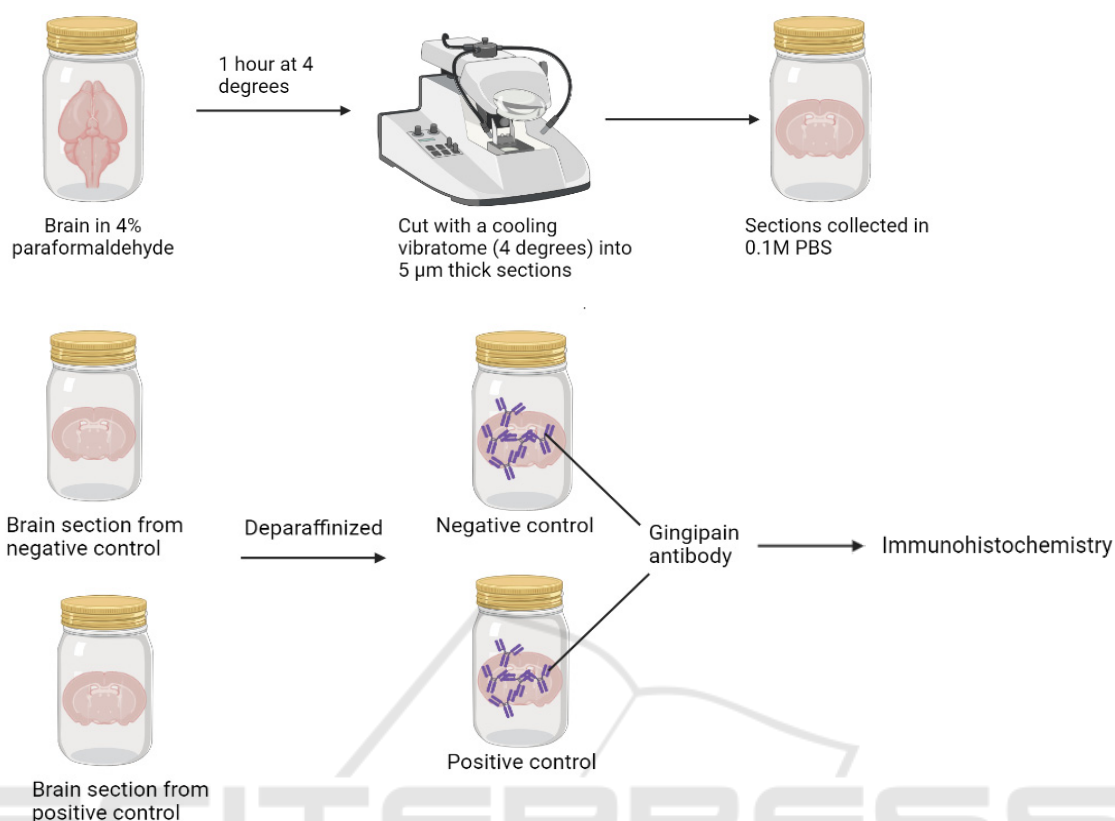


Figure 3: Steps of making brain sections for immunohistochemistry.

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