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Original Research

Systemic Administration of *Porphyromonas Gingivalis* Lipopolysaccharide Induces Glial Activation and Depressive-Like Behavior in Rats

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Abstract

Background: Periodontitis is one of the most common chronic inflammatory disorders in adults. Although clinical studies have suggested a causal relationship between periodontitis and major depression (MD), the biological mechanisms by which periodontitis instigates MD are unknown. We investigated whether a systemic administration of lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (*Pg*), a major Gram-negative pathogen of periodontitis, causes depressive-like behavior and glial activation in the hippocampus and the prefrontal cortex (PFC), which are MD-related brain regions. **Materials and Methods**: Eight-week-old male Sprague Dawley rats were randomly divided into a behavioral test group and an immunohistochemistry group. The rats in each group were further assigned to the sham injection (saline) and *Porphyromonas gingivalis*-lipopolysaccharide (*Pg*-LPS) injection protocols. The rats received an intraperitoneal injection of saline or *Pg*-LPS with gradually increasing doses (day 1: 0.5, day 2: 0.5, day 3: 0.75, day 4: 0.75, day 5: 1.0, day 6: 1.0, and day 7: 1.0 mg/kg of body weight) for seven consecutive days. After the systemic administration, the behavior test group underwent the forced swimming test (FST) and Y-maze test. For the immunohistochemistry group, we quantified the immunoreactivity for microglial Iba-1 (ionized calcium-binding adapter molecule 1) and astrocytic glial fibrillary acidic protein (GFAP) in the hippocampus (dentate gyrus [DG], cornu ammonis [CA1 and CA3]) and PFC (prelimbic [PrL] and the infralimbic [IL]) areas. **Results**: The FST immobility time in the *Pg*-LPS group was significantly longer than that in the sham group. In the Y-maze test, a significant decline in spontaneous alternation behavior was observed in the *Pg*-LPS group compared to the sham group. The peripheral administration of *Pg*-LPS significantly increased the immunoreactivity for Iba-1 in the CA3 and PrL. *Pg*-LPS injection significantly increased the immunoreactivity for GFAP in the DG, CA1, and CA3. **Conclusions**: The major result of this study is that a repeated systemic administration of *Pg*-LPS caused depressive-like behavior and both microglial and astrocytic activation in rats. This finding may comprise biological evidence of a causal relationship between periodontitis and MD.

Keywords: microglia; astrocytes; glial activation; *Porphyromonas gingivalis*; lipopolysaccharide; periodontitis; depressive-like behavior

1. Introduction

Major depression (MD) is a prevalent life-threatening psychiatric disorder. Approximately 322 million people are affected by MD worldwide [1]. The neuroinflammatory process in which activated microglia and astrocytes generate excessive pro-inflammatory cytokines has been implicated in the pathogenesis of MD [2–4]. Increasing evidence also suggests that infection and persistent low-grade inflammation in peripheral tissues play a role in MD [5]. Periodontitis is one of the most common chronic inflammatory diseases in adults [6–8]. Although recent systematic reviews and meta-analyses have suggested a mutual relationship between periodontitis and MD [9–13], the biological mechanisms by which periodontitis instigates MD are unclear.

Several animal studies have demonstrated that peripheral administration of lipopolysaccharide (LPS) from *Escherichia coli* increases the levels of pro-inflammatory cytokines in both the periphery and brain and induces abnormal behavior similar to MD, called depressive-like behavior [14–17]. It has also been believed that systemic inflammation causes the neuroinflammatory process through sev-

eral pathways [5]. It is proposed that such neuroinflammation, in turn, elicits MD symptoms by influencing brain functions, especially neurotransmitter metabolism [18]. We conducted the present study to determine whether repeated systemic inflammation induced by intraperitoneal (i.p.) injection of LPS from *Porphyromonas gingivalis* (*Pg*), a major Gram-negative pathogen associated with periodontitis, causes depressive-like behavior and glial activation in the rat hippocampus and the prefrontal cortex (PFC), which are MD-related brain regions. Previous rodent studies along this line have demonstrated that i.p. injection of *Porphyromonas gingivalis*-lipopolysaccharide (*Pg*-LPS) causes depressive-like behavior [19] and cognitive dysfunction [20] with glial activation.

2. Materials and Methods

2.1 Animals

Eight-week-old male Sprague Dawley (SD) rats (SLC, Inc., Shizuoka, Japan) were used. They were housed in plastic cages at a room temperature of 23 $\pm$ 2 °C, 12-hr light/12-hr dark cycle (lights on from 7:00–19:00), and 55 $\pm$ 5% humidity with free access to food and water. One week before the start of the experiment, the rats underwent a handling procedure once daily to decrease the experimental stress. The handling method consisted of lifting the rat with a gloved hand and massaging it for 10 minutes. All experimental procedures were conducted and approved by the Shimane University Animal Ethics Committee (Authorization No: IZ3-50).

2.2 Chemicals

Pg-LPS was purchased from InvivoGen (catalog no. tlrl-pglps, version no. 14F18-MM, San Diego, CA, USA). *Pg*-LPS was dissolved in endotoxin-free water.

2.3 Treatment Procedure

We randomly divided the SD rats into a behavioral test group and an immunohistochemistry group. Each group was further assigned to the sham injection and *Pg*-LPS injection groups. It has been documented that the stress intensity of the forced swimming test (FST) is very strong [21], and the FST stress has the possibility of affecting brain structure and function [22]. In order to avoid such influence on immunohistochemistry results, we divided rats into the group undergoing behavior tests only and the group undergoing the immunohistochemical analysis only. The number of individuals in each group was 5 or 6. Investigators were not blinded to the group allocation at any stage.

The rats in the *Pg*-LPS subgroup received *Pg*-LPS at gradually increasing doses by daily i.p. injection for seven consecutive days (Fig. 1). The *Pg*-LPS dose was increased as follows: day 1: 0.5, day 2: 0.5, day 3: 0.75, day 4: 0.75, day 5: 1.0, day 6: 1.0, and day 7: 1.0 (mg/kg of body weight (BW)). Repeated daily administration of increasing LPS doses has been shown to induce depressive-like behavior more robustly, compared to administration of consistent LPS dose, with avoidance of LPS tolerance [15].

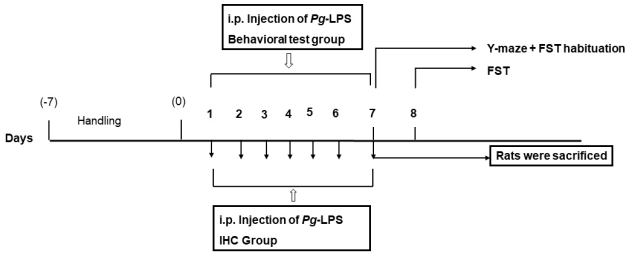

Fig. 1. The experimental schedule. *Pg*, *Porphyromonas gingivalis*; LPS, Lipopolysaccharide; i.p., intraperitoneal; IHC, immunohistochemistry; FST, forced swimming test.

The sham injection group received an equivalent volume of saline intraperitoneally and daily for seven consecutive days. In the behavioral test group, the Y-maze test was performed 2 hr after the last i.p. injection. 2 hr after the Y-maze test, habituation of the forced swimming test (FST) was carried out. In the immunohistochemistry group, brain samples were collected 2 hr after the last injection (Fig. 1).

2.4 Forced Swimming Test

The forced swimming test (FST) was performed as described [23]. Briefly, the rat was placed in a cylinder of water for 6 min, and its behavior was recorded by a video camera. The time that the rat spent in an immobile state (i.e., the immobility time) throughout the test was manually counted based on the recorded video. The rat was judged immobile when it persisted motionless and floating without struggling and when it moved merely to maintain its head above the water.

2.5 Y-Maze Test

The Y-maze test assessed the spatial working memory after the last *Pg*-LPS injection following a described protocol [23]. Briefly, the rat was placed at the terminus of one arm of the maze apparatus (catalog no.YM-03R, Muromachi Kikai, Tokyo, Japan) and allowed to move freely across the maze for 8 min. The entry was regarded as complete when the tail of the rat was entirely within the arm. Alternation behavior was regarded as successive entries into all three arms of the maze on successive occasions. The percentage of spontaneous alternation behavior (SAB) was estimated by the formula mentioned below.

$$\%\text{SAB} = \frac{\text{The number of alternations}}{\text{The total number of arms entries} - 2} \times 100\%$$

2.6 Brain Section Preparation

At 2 hr after the last administration of the *Pg*-LPS treatment, the rats in the immunohistochemistry group

were sacrificed under deep intraperitoneal anesthesia with a mixture of three drugs: medetomidine (Domitor, Nippon Zenyaku Kogyo, Fukushima, Japan) at 0.15 mg/kg BW/rat, midazolam (Dormicum, Astellas Pharma, Tokyo, Japan) at 2 mg/kg BW/rat and butorphanol (Vetorphale, Meiji Seika Pharma, Tokyo, Japan) at 2.5 mg/kg BW/rat. Saline (Otsuka Pharmaceutical Factory, Tokushima, Japan) was then added up to adjust the mixture to a volume of 0.5 mL/100 g BW/rat. The rats were perfused transcardially with 500 mL of physiological saline and then 500 mL of 4% of paraformaldehyde (PFA) in phosphate buffer solution (Fujifilm, Wako, Osaka, Japan). After the perfusion, the brains were quickly removed and fixed with 4% of paraformaldehyde in phosphate buffer solution for 4 hr at room temperature (RT). The brains were submersed in 10% sucrose solution overnight at 4 °C and were subsequently immersed in 20% sucrose solution for 24 hr at 4 °C. The brains were sectioned at 40-µm thickness in the frontal plane by using a microtome (Microm HM 430, Thermo Scientific, Walldorf, Germany).

2.7 Immunohistochemistry

The free-floating brain sections were incubated in 1% H_2O_2 at RT for 30 min, followed by blocking incubation with 0.2% Triton-X and 1.5% and normal horse serum in 0.1 M phosphate buffer at RT for 1 hr. The brain sections were incubated overnight at RT with rabbit anti-ionized calcium-binding adapter molecule (Iba)-1 antibody (1:2000, catalog no. 019-19741, Wako, Osaka, Japan) and goat anti-glial fibrillary acidic protein (GFAP) antibody (1:2000, Abcam, Cambridge, UK). The next day, the sections were treated with biotinylated anti-rabbit or anti-goat IgG antibody (1:1000, catalog no. PK-4000, standard ABC kit, Vector Laboratories, Newark, CA, USA) at RT for 1 hr. The sections were incubated at RT for 1 hr with an avidin-biotin-peroxidase complex in phosphate-buffered saline (PBS). The immunoreactivity in the brain tissues was developed by incubating in PBS comprising 0.5% diaminobenzidine (DAB) and 0.03% H_2O_2 for 10 min. To halt the DAB reaction, the brain sections were washed with PBS for 30 min.

2.8 Quantification of Immunoreactivity

The intensity of microglial and astrocytic immunoreactivity in DAB-stained images was measured by a computer-assisted image analysis program (ImageJ 1.52a, National Institutes of Health, Bethesda, MD, USA) described [24]. The images were captured by using a BZ-X700 all-in-one microscope (Keyence, Osaka, Japan) with a 20× objective lens. The regions of interest consisted of three areas within the hippocampus, i.e., the dentate gyrus (DG), the cornu ammonis 1 (CA1), and the CA3 and two areas within PFC, i.e., the prelimbic (PrL) and the infralimbic (IL) areas. Twenty images were captured bilaterally from each area of the hippocampus (10 images from the left hemisphere and 10 images from the right hemisphere). Sixteen images were captured bilaterally from each area of the PFC (8 images from the left hemisphere and 8 images from the right hemisphere). A total of 92 images (60 images of hippocampus and 32 images of PFC) were analyzed on every rat (n = 5). The software automatically translated all immunolabeled components beyond the threshold range into black pixels and translated the rest of the image into white pixels. The software estimated the percentage of black pixels for statistical analysis.

2.9 Statistical Analysis

All the data are expressed as the mean ± standard deviation (S.D.). The experimental unit of analysis is the SD rat. For two group comparisons, we performed the independent sample *t*-test. The primary outcome is the % of SAB in the Y-maze test. The secondary outcomes are the immobility time in the FST and % of black pixel area in immunohistochemical analysis. The minimum sample size was calculated as n = 5 with the Power*G software (version.3.1.9.2, Heinrich-Heine University, Dusseldorf, Germany). The parameters, which comprise effect size d = 2.437, α error prob = 0.05; power (1-β error prob) = 0.9, and allocation ratio = 1, were put into the software. Using the Shapiro-Wilks test, we confirmed that the data met the statistical assumptions. The statistical analysis was conducted by using the software SPSS (version. 23.0, IBM Corp., Tokyo, Japan). A *p*-value less than 0.05 was considered significant.

3. Results

3.1 Behavioral Tests

The behavioral tests were conducted to examine whether peripheral injection of *Pg*-LPS can cause depressive-like behavior, such as behavioral despair and impaired spatial working memory. In the FST, learned helplessness in the rats was evaluated by measuring the immobility time, which was 108.73 ± 29.33 sec in the sham group (n = 6) and 181.54 ± 49.20 sec in the *Pg*-LPS group (n = 5) (Fig. 2A). The immobility time in the *Pg*-LPS group was significantly longer than that in the sham group, suggesting that the i.p. injection of *Pg*-LPS induce behavioral despair (Table 1).

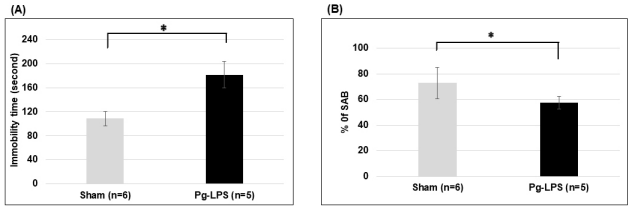

Fig. 2. Behavioral tests of *Pg*-LPS-injected rats. (A) Effects of *Pg*-LPS injection on immobility time in the forced swimming test. (B) Effect of *Pg*-LPS injection on spontaneous alternation behavior (SAB) in the Y-maze test. *$p < 0.05$. Each value is the mean ± S.D. Sham group (n = 6), *Pg*-LPS group (n = 5).

Table 1. Results of behavioral tests.

	Group	Mean ± S.D.	*p*-value
Immobility time (sec)	Sham (n = 6)	108.73 ± 29.33	0.014
	Pg-LPS (n = 5)	181.54 ± 49.20	
% SAB	Sham (n = 6)	72.83 ± 12.29	0.028
	Pg-LPS (n = 5)	57.43 ± 4.75	

S.D., standard deviation; SAB, spontaneous alternation behavior; *Pg*-LPS, *Porphyromonas gingivalis*-lipopolysaccharide.

Table 2. Immunoreactivity for Iba1 in the hippocampus and prefrontal cortex.

	Group	% of black pixel area (Mean ± S.D.)	*p*-value
DG	Sham (n = 5)	16.09 ± 3.70	N.S.
	Pg-LPS (n = 5)	19.92 ± 3.07	
CA1	Sham (n = 5)	18.82 ± 2.30	N.S.
	Pg-LPS (n = 5)	20.79 ± 1.77	
CA3	Sham (n = 5)	13.95 ± 1.57	0.014
	Pg-LPS (n = 5)	18.05 ± 2.49	
PrL	Sham (n = 5)	13.49 ± 2.31	0.008
	Pg-LPS (n = 5)	18.05 ± 1.73	
IL	Sham (n = 5)	12.86 ± 2.88	N.S.
	Pg-LPS (n = 5)	16.65 ± 3.29	

S.D., standard deviation; DG, dentate gyrus; *Pg*-LPS, *Porphyromonas gingivalis*-lipopolysaccharide; CA, cornu ammonis; PrL, prelimbic; IL, infralimbic; N.S., not significant.

We evaluated the rat's spatial working memory in the Y-maze test by calculating the percentage of SAB. As shown in Fig. 2B. the percentage of SAB was 72.83 ± 12.29% in the sham group (n = 6) and 57.43 ± 4.75% in the *Pg*-LPS group (n = 5). A significant reduction in the % SAB was detected in the *Pg*-LPS group compared to the sham group. This result suggests that the i.p. injection of *Pg*-LPS impaired spatial working memory (Table 1).

3.2 Immunohistochemical Analysis

We investigated the immunoreactivity of microglial Iba-1 in the hippocampus (CA1, CA3, and DG regions; Fig. 3A,B,D,E,G,H) and the PFC (PrL and IL regions; Fig. 3J,K,M,N). Among these regions, the CA3 region showed significantly higher immunoreactivity for Iba-1 in the *Pg*-LPS-injection group (18.05 ± 2.49%, n = 5) compared to the sham injection group (13.95 ± 1.57%, n = 5) (Fig. 3I). Also, the PrL region showed significantly higher immunoreactivity for Iba-1 in the *Pg*-LPS-injection group (18.05 ± 1.73%, n = 5) compared to the sham injection group (13.49 ± 2.31%, n = 5) (Fig. 3L). In the other regions, the *Pg*-LPS group (n = 5) showed a non-significant trend for higher Iba-1 immunoreactivity compared with the sham group (n = 5) (Fig. 3C,F,O) (Table 2).

We also evaluated the astrocytic GFAP immunoreactivity in the hippocampus (CA1, CA3 and DG regions; Fig. 4A,B,D,E,G,H) and the PFC (PrL and IL regions; Fig. 4J,K,M,N). The hippocampal CA1 regions showed significantly higher immunoreactivity for GFAP in the *Pg*-LPS-injection group (27.98 ± 2.69%, n = 5) compared to the sham injection group (21.75 ± 4.88%, n = 5) (Fig. 4F) and CA3 regions showed significantly higher immunoreactivity for GFAP in the *Pg*-LPS-injection group (21.47 ± 1.13%, n = 5) compared to the sham injection group (14.41 ± 3.21%, n = 5) (Fig. 4I). Also the DG region showed significantly higher immunoreactivity for GFAP in the *Pg*-LPS-injection group (31.19 ± 2.10%, n = 5) compared to the sham injection group (22.59 ± 4.67%, n = 5) (Fig. 4C). On the other hand, in the PFC PrL and IL regions, we observed no significant difference in GFAP immunoreactivity between the *Pg*-LPS (n = 5) and sham groups (n = 5) (Fig. 4L,O) (Table 3).

4. Discussion

A major finding from the behavioral tests in the present study is that repeated i.p. injection of *Pg*-LPS elicited depressive-like behavior in SD rats. In the FST, the *Pg*-LPS-injected rats showed significantly longer immobility time compared to saline-injected rats. The immobility time represents learned helplessness and can be considered behavioral despair [22,25]. This finding is consistent with an earlier investigation which demonstrated that mice treated intraperitoneally with *Pg*-LPS exhibited significantly prolonged immobility time in the FST [19]. The

Table 3. Immunoreactivity for GFAP in the hippocampus and the prefrontal cortex.

Area	Group	% of black pixel area (Mean ± S.D.)	*p*-value
DG	Sham (n = 5)	22.59 ± 4.67	0.006
	Pg-LPS (n = 5)	31.19 ± 2.10	
CA1	Sham (n = 5)	21.75 ± 4.88	0.037
	Pg-LPS (n = 5)	27.98 ± 2.69	
CA3	Sham (n = 5)	14.41 ± 3.21	0.002
	Pg-LPS (n = 5)	21.47 ± 1.13	
PrL	Sham (n = 5)	10.06 ± 4.00	N.S.
	Pg-LPS (n = 5)	14.56 ± 3.09	
IL	Sham (n = 5)	10.37 ± 4.05	N.S.
	Pg-LPS (n = 5)	14.28 ± 3.93	

S.D., standard deviation; DG, dentate gyrus; *Pg*-LPS, *Porphyromonas gingivalis*-lipopolysaccharide; N.S., not significant.

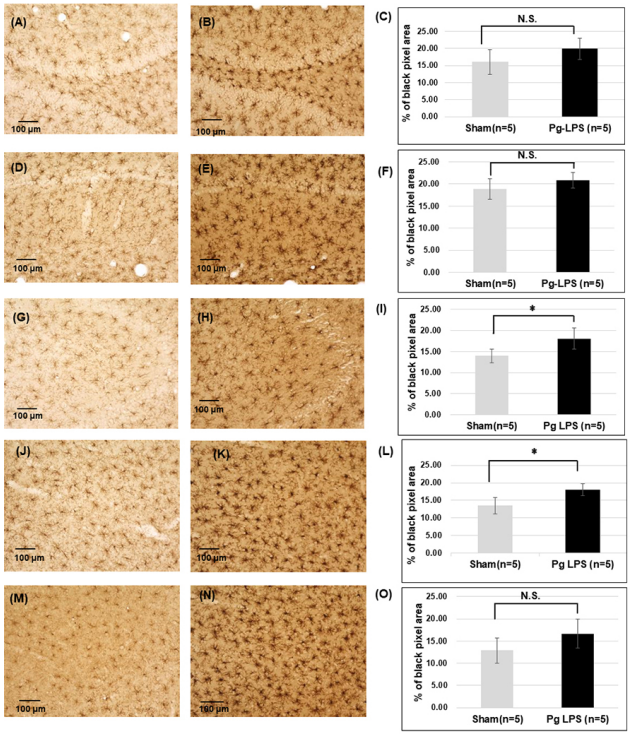

Fig. 3. Immunoreactivity for Iba1 in the hippocampus and prefrontal cortex. Representative images of 0.5% diaminobenzidine (DAB) staining in the dentate gyrus (DG) of Sham (A) and *Pg*-LPS rats (B), the cornu ammonis (CA)1 of Sham (D), and *Pg*-LPS rats (E), and CA3 of the Sham (G) and *Pg*-LPS rats (H), prelimbic (PrL) area of Sham (J) and *Pg*-LPS rats (K), and the infralimbic (IL) area of Sham (M), and *Pg*-LPS rats (N). Scale bars, 100 μm; Quantification data of Iba1-immunoreactivity in the DG (C), CA1 (F), CA3 (I), PrL (L), and IL (O). *$p < 0.05$. N.S., not significant. Each value is the mean ± S.D. (n = 5).

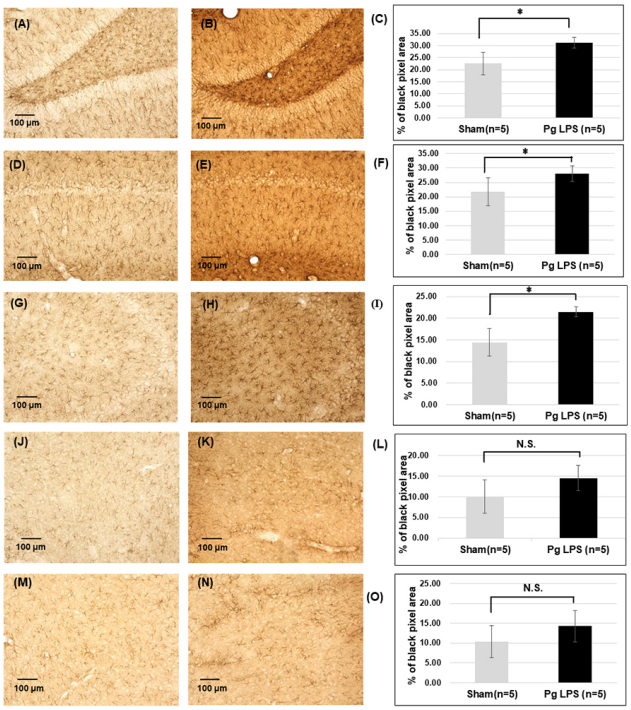

Fig. 4. Immunoreactivity for GFAP in the hippocampus and the prefrontal cortex. Representative images of DAB staining in the DG of Sham (A) and *Pg*-LPS rats (B), CA1 of Sham (D) and *Pg*-LPS rats (E), CA3 of Sham (G) and *Pg*-LPS rats (H), PrL of Sham (J) and *Pg*-LPS rats (K), and IL of Sham (M), and *Pg*-LPS rats (N). Scale bars, 100 μm; Quantification data of Iba1-immunoreactivity in the DG (C), CA1 (F), CA3 (I), PrL (L), and IL (O). *$p < 0.05$. N.S., not significant. Each value is the mean ± S.D. (n = 5). GFAP, glial fibrillary acidic protein.

Pg-LPS-injected rats in the present study also showed a significantly decreased % of SAB in the Y-maze test. The decrease in SAB can be interpreted as impaired spatial working memory [26,27]. Clinically, it has been observed that individuals with MD tend to have considerably impaired working memory [28]. Our present finding is also in line with a report that peripheral injection of *Pg*-LPS led to impaired spatial learning in mice [20]. The previous investigations of the effects of *Pg*-LPS on depressive-like behavior used mice [19,20]; to the best of our knowledge, the present

study is the first one in rats demonstrating that a peripheral administration of *Pg*-LPS induced behavioral despair and impaired spatial working memory.

A major finding of this study's immunohistochemical analysis is that the systemic administration of *Pg*-LPS increased microglial Iba-1 immunoreactivity and astrocytic GFAP immunoreactivity in the MD-related brain regions, i.e., the hippocampus and PFC. Our finding of astrocytic activation in the hippocampal regions, as shown by raised GFAP immunoreactivity, is consistent with the above-cited studies using mice, which demonstrated that i.p. injection of *Pg*-LPS raised the number of GFAP-immunopositive cells in the hippocampus and PFC [19,20]. We also observed a non-significant trend towards an increase in GFAP immunoreactivity in the PFC. Accordingly, the hippocampus may be more sensitive to the activation of astrocytes by *Pg*-LPS compared to the PFC.

Our finding of *Pg*-LPS-induced activation of microglia in the CA3 and PrL regions was verified by quantification of Iba-1 immunoreactivity, while an aforementioned study [20] described microglial activation based only on morphological inspection. The other study mentioned above [19] reported no significant change in the messenger RNA (mRNA) of the microglial markers Iba-1 and CX3CR1 in *Pg*-LPS-injected mice. Such discrepancy in the effects of *Pg*-LPS on the microglial state may stem from differences in the species and/or the concentrations of *Pg*-LPS applied.

It has been documented that *E.coli*-LPS containing lipid A with a conical shape induces the production of proinflammatory cytokines through Toll-like receptor (TLR) 4. On the other hand, *Pg*-LPS comprising more cylindrical lipid A, binds to TLR2 and elicits the cytokine production less efficiently than *E.coli*-LPS [29]. Moreover, *Pg*-LPS has been shown to induce a weaker neuroinflammatory response, compared with *E.coli*-LPS, in the rat circumventricular organs [30]. We, therefore, used higher doses of *Pg*-LPS in this study, relative to typical *Pg*-LPS doses (0.1–0.2 mg/kg) capable of causing depressive-like behavior in rats [31,32].

It is elusive whether glial activation is exactly involved in the MD pathogenesis, as only a few studies have evaluated glial activation using postmortem brain samples from individuals with MD. Nevertheless, *in vivo,* studies demonstrated that mice with activated microglia and activated astrocytes, which were induced by peripheral injection of *E. coli*-LPS, exhibited depressive-like behavior [3, 33]. Our present finding verified that *Pg*-LPS also caused microglial activation, astrocytic activation, and depressive-like behavior in rats. Moreover, the results of one of our earlier investigations implied that both microglial activation and astrocytic activation in the hippocampus were related to depressive-like behavior in Gunn rats with congenital hyperbilirubinemia, another neuroinflammatory model associated with MD [34]. Taken together, our past and present findings support the hypothesis that glial activation may be closely correlated with presenting MD symptoms.

It has been suggested that dissociation between sickness behavior and depressive-like behavior attributes to time course in LPS-injected rodents [35]. Their sickness behavior, which can be considered neurovegetative symptoms (i.e., loss of appetite, insomnia, and fatigue), emerges first in response to LPS injection. Depressive-like behavior, which can be regarded as mood and cognitive symptoms, develops after the expression of sickness behavior. The limitation of this study is that changes in BW and sleeping time, which seem to reflect sickness behavior, were not evaluated.

Other limitations of the present study are as follows: First, we did not validate the immunohistochemistry findings via other methods, including gene expression profiling. Second, we did not perform behavioral tests on every rat. Third, *Pg*-LPS was injected into the peritoneal cavity, not into the gingiva. Accordingly, the experimental condition in this study does not exactly mimic periodontitis.

5. Conclusions

Our major finding is that a repeated systemic administration of *Pg*-LPS elicited depressive-like behavior, and both microglial and astrocytic activation could be biological evidence for a causal relationship between periodontitis-associated pathogens and MD.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

RM: data curation, formal analysis, writing original draft, validation. SH: supervision, writing manuscript, data curation, validation, funding acquisition, project administration. IAA: data curation, validation. MAJ: data curation, validation. SJFJ: data curation, validation. KKK: validation. JF: data curation. KI: statistical analysis. MI: data curation. HT: supervision, validation. All authors contributed to editorial changes in the manuscript. All authors have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures were conducted and approved by the Shimane University Animal Ethics Committee, according to the guidelines of the National Health and Medical Research Council of Japan (Authorization No: IZ3-50).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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