Porphyromonas gingivalis induces depression via downregulating p75NTR-mediated BDNF maturation in astrocytes

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ABSTRACT
Many cross-sectional epidemiological studies have shown the incidence of periodontitis is positive correlated with that of depression. However, their causal relationship and underlying mechanism are largely unknown. Porphyromonas gingivalis (Pg) is the main pathogen for periodontitis. Employing female mice treated with Pg every other day for 4 weeks, we found that Pg-mice showed obvious depression-like behavior, an increased number of activated astrocytes and decreased levels of mature brain derived neurotrophic factor (BDNF) and astrocytic p75NTR in the hippocampus. Both hippocampal injection of BDNF and overexpression of p75NTR in astrocytes alleviated Pg-induced depression-like behavior in mice. Moreover, Pg-lipopolysaccharides (LPS) generated similar phenotypes, which were reversed by the TLR-4 inhibitor TAK242. Our results suggest that Pg-LPS decreases the level of astrocytic p75NTR and then downregulates BDNF maturation, leading to depression-like behavior in mice. Our study provides the first evidence that Pg is a modifiable risk factor for depression and uncovers a novel therapeutic target for the treatment of depression.

1. Introduction
Depression is a psychological disorder characterized by prominent morbidity and mortality, and its incidence is dramatically increasing (Gobbi et al., 2019; Health Quality, 2017). Many cross-sectional studies have shown a positive correlation between periodontitis and depression (Hsu et al., 2015; Ng and Keung Leung, 2006). However, the causal relationship and relative mechanism between these diseases are still unknown.

Pg is a Gram-negative anaerobic pathogen of periodontitis (Jiao et al., 2014). Pg produces LPS, gingipain, and capsule that damage local periodontal tissue (Mysak et al., 2014) and also affect many other organs via systemic circulation (Teixeira et al., 2017; Zhang et al., 2018). It has been reported that Pg is positively correlated with Alzheimer’s disease (Dominy et al., 2019). However, whether it contributes to the pathogenesis of depression is still unclear. LPS usually activates TLR-4-mediated immune responses, promotes release of inflammatory factors by immune cells, and affects various body systems (Blasco-Baque et al., 2017; Zhang et al., 2018). Further, LPS has been detected in the brain (Zhan et al., 2016), and regulates synaptic formation and synaptic plasticity in an astrocyte-dependent manner (Shen et al., 2016).

The neurotrophin deficiency hypothesis of depression has received extensive attention in recent years (Huang and Reichardt, 2003). This hypothesis states that decreased neurotrophic factors render the brain unable to adapt to environmental stimulation, which contributes to the onset of depression (Duman, 2002). Brain derived neurotrophic factor (BDNF) is an important member in the neurotrophin family and plays a pivotal role in formation and plasticity of neuronal networks (Huang and Reichardt, 2003). It has been reported that BDNF levels are reduced in the hippocampus of postmortem tissue from suicide victims and in the serum of depressive patients (Autry and Monteggia, 2012). Additionally, BDNF is upregulated by treatment with antidepressant drugs in patients (Duman et al., 2001). infusion of BDNF into the midbrain or hippocampal dentate gyrus (DG) results in increased antidepressant-like behavior in mice (Hu and Russek, 2008).

Mature BDNF (mBDNF) can be produced by two different ways. After a 32 kDa BDNF precursor protein (proBDNF) is synthesized in the cytoplasm, it is secreted from the cytoplasm and cleaved by...
metalloproteinases directly or taken up by neurotrophic factor receptor p75 (p75NTR) and then cleaved by furin and proconvertase (Bergami et al., 2008). Expression of p75NTR not only rhythmically fluctuates under physiological conditions (Shu et al., 2015) but also makes adaptive change under pathological conditions. For example, expression of p75NTR increases significantly in an animal model with ischemia and epilepsy and the molecular mechanism is still unclear (Oderfeld-Nowak et al., 2003). In addition, it has been proposed that p75NTR contributes to the development of depression by dysregulating BDNF maturation (Shu et al., 2015).

Astrocytes play an important part in the pathological process of depression through regulating glutamate homeostasis, energy metabolism and neurotrophic support (Wang et al., 2017). Changes in the number and activity of astrocytes can be detected in patients with depression (Rajkowska and Stockmeier, 2013). Overexpression of BDNF in astrocytes is sufficient to rescue depression and anxiety-like behaviors in mice (Quesseveur et al., 2013). Moreover, astrocytes express high levels of p75NTR, which plays a role in BDNF maturation (Cragnolini et al., 2009). Thus, it is logical to explore the role of astrocytic p75NTR in the pathogenesis of Pg-induced depression.

To demonstrate the relationship between Pg and depression, we established a mouse model of periodontitis through oral colonization of Pg and investigated its role in the pathogenesis of depression. We found that Pg induced depression-like behaviors proportional to Pg-induced alveolar bone loss. In the brain of Pg-treated mice, astrocytic activity was increased, while astrocytic p75NTR and mBDNF levels were reduced. These results highlight a previously unexplored role of Pg in the onset of depression. The potential mechanism is through Pg-LPS downregulation of p75NTR expression in astrocytes which impairs BDNF maturation and results in depression.

2. Materials and methods

2.1. Mice

All experimental protocols were approved by the Institutional Animal Care and Use Committee of South China Agricultural University (approval ID: 2017-C011), and all experiments were carried out in accordance with National Institutes of Health guide for the care and use of laboratory animals. Female C57BL/6J wild-type mice were purchased from Guangzhou University of Chinese Medicine. hGFAP-Cre mice, which generate Cre recombinase under the control of the human glial fibrillary acidic protein promoter, were kindly provided by Professor Yanmei Tao’s Lab in Hangzhou Normal University. The mice were housed in groups (six mice per cage) in a specific pathogen-free environment (inverted 12 h daylight cycle, light off at 10:00) at room temperature (22 ± 2 °C). Food and water were provided ad libitum. Mice were raised under the standards of the Division of Laboratory Animals, South China Agricultural University.

2.2. Oral colonization

The murine model of periodontitis we used was first described by Baker et al. (2000) and modified (Blasco-Baque et al., 2017; Dominy et al., 2019). Six-week-old mice were randomized into three groups: group 1 and group 2 were colonized with Pg ATCC 33277 and Fusobacterium nucleatum (Fn) ATCC 10953, respectively. For group 1 and group 2, we applied 1 mL of a mix of 10^9 colony forming units (CFU) of Pg or Fn in 2% carboxymethylcellulose on the surface of the mandibular molar teeth every other day for 4 weeks. Group 3 served as a control and received vehicle only.

2.3. Evaluation of mandibular alveolar bone resorption

We scanned hemi-mandibles with micro CT (μCT 50, SCANCO Medical AG, Swiss). Data were acquired at 70 kV with a 10 μm isotropic voxel size. Three dimensional reconstructions were generated from a set of 700–800 slices per mandible with Mimics Research 19.0 (Materialise, USA). Linear measurements were obtained in the first molar to determine alveolar bone loss (ABL), which was determined from the alveolar bone crest to the cemento-enamel junction.

2.4. Evaluation of depression-like behavior

2.4.1. Tail-suspension test (TST)

We suspended the mice with tapes around tail ends for 6 min and recorded the immobile durations during the last 4 min. The latency to immobility was recorded as the first time at which the mice became immobile.

2.4.2. Forced-swim test (FST)

One day before the test, we placed the mice in plastic buckets with water at a depth of 23 cm for 15 min, after which the mice were instantly dried with clean towels and warmed with a heating lamp. On the test day, mice were placed into the same containers for 6 min. The final 4 min were monitored for immobility duration. The time at which the mice first became immobile was recorded as the latency to immobility. Immediately following the test, mice were gently dried with clean towels and placed under heating lamps.

2.5. Anxiety-like behavior evaluation

2.5.1. Open-field test (OFT)

We performed OFT in a square box which was divided into outer and inner zones. Mice were placed in the center position and scored for 30 min with a video tracking system (JLBhv-LAM-4, Jiliang Software Technology, Shanghai, China).

2.5.2. Elevated plus maze test (EPM)

EPM consisted of two open and two closed arms which had high walls at both sides and the end. We placed each mouse in the central square (10 × 10 cm) facing an open arm and recorded the mouse exploring the maze for 10 min with a tracking system (JLBehv-LAM-4, Jiliang Software Technology, Shanghai, China). The amount of time spent in the open arms and closed arms was recorded.

2.6. Elisa analysis

We collected blood in vacutainer tubes. The tubes were centrifuged at 12,000 rpm for 15 min. We collected supernatant in 1.5 mL Eppendorf tubes. Corticosterone levels were determined with ELISA kits (R&D Systems, USA) following the manufacturer’s instructions.

After the mice were sacrificed, brain tissue was quickly removed, and the prefrontal cortex and hippocampus were carefully isolated with microscopic forceps. The isolated tissue was homogenized in PBS (pH 7.4) before centrifugation. The supernatant was collected and used as the test sample. We quantified the protein levels with the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Vernon Hills, IL, USA). TNF-α, IL-6, and IL-1α (Arigo Biolaboratories, Hsinchu City, Taiwan, China) levels were determined using ELISA kits following the manufacturer’s instructions.

2.7. RNA-seq

The prefrontal cortex and hippocampus were carefully isolated using microscopic forceps from the brain. RNA-Seq was utilized to produce approximately 10,000 sequences in every sample, and data were analyzed by Vazyme Biotech (Jiangsu, China). To detect the quality of total RNA, mRNA was purified and fragmented, double stranded cDNA was synthesized, termini were repaired, A was added, connectors were added, fragment sizes were sorted, the library was amplified, quality detection was performed, and machine sequencing
was performed. After obtaining the original sequencing data of the samples, biological information was analyzed according to the reference sequences related to depression in the NCBI National Center for Biotechnology Information.

2.8. Histological analysis

Mice were anesthetized with phenobarbital sodium (4.5 mL/kg) and perfused with physiological saline and 4% paraformaldehyde. Hemimandibles were excised, fixed in 4% paraformaldehyde for 48 h and embedded in paraffin. Hemimandibles samples were cut with a microtome in the transverse direction following the main axis of tooth from coronal to apical. Then, sections (4 mm thickness) were stained with Hematoxylin and Eosin (H&E) staining kit (CO105, Beyotime). Slides were scanned with a panoramic digital scanner Aperio AT2 (Leica, Germany).

We also immediately removed the brain, placed the brain in 4% paraformaldehyde and left it overnight at 4 °C. Then, the brains were subjected to gradient dehydration with 10%, 20%, and 30% sucrose solutions. Coronal sections that were 20 μm thick and contained the prefrontal cortex or hippocampus were made using a freezing microtome (CryoStar NX50, Thermo Fisher Scientific). We incubated the sections with the following primary antibodies overnight at 4 °C: mouse anti-β-gal (1:500, ab203294; Abcam), anti-p75NTR (1:1000, no. 4504; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-BDNF (1:1000, ab13867; Abcam, Cambridge, UK), anti-proBDNF (H + L) (1:200, no.33106ES60; Yeason, China), Alexa Fluor 488 AffiniPure Goat Anti-Mouse IgG (H + L) (1:200, no.35206ES60; Yeason, China), and Alexa Fluor 488 AffiniPure Goat Anti-Rabbit IgG (H + L) (1:200, no.32122ES60; Yeason, China), and Alexa Fluor 488 AffiniPure Goat Anti-Rabbit IgG (H + L) (1:200, no.33106ES60; Yeason, China). Sections were then stained with 4′,6-diamidino-2-phenylindole (DAPI) staining solution (no. E607303; Yeason), and Alexa Fluor 488 AffiniPure Goat Anti-Mouse IgG (H + L) (1:200, no.32122ES60; Yeason, China), and Alexa Fluor 488 AffiniPure Goat Anti-Rabbit IgG (H + L) (1:200, no.33106ES60; Yeason, China). Sections were then stained with 4′,6-diamidino-2-phenylindole (DAPI) staining solution (no. E607303; Sangon Biotech). Slides were scanned with an LSM780 (ZEISS, Germany) using ZEN 2.3 imaging software (ZEISS) with the z-stack function, and images were analyzed with Image-Pro Plus 6.0 (Media Cybernetics, USA). Fifteen slides for each group were counted by two independent investigators.

2.9. Western blot

Hippocampal samples were homogenized in RIPA lysis buffer with 1% protease inhibitor cocktail and 1% PMSF. The samples were denatured via boiling for 5 min after adding sodium dodecyl sulfate (SDS) (loading buffer, and proteins were transferred to polyvinylidene difluoride (PVDF) membranes following separation. The membrane was blocked with skimmed milk. Pg lipopolysaccharide (LPS) was probed with a monoclonal antibody (MAB 185) which was kindly supplied by Professor Mike Curtis (Curtis et al., 1999). Probes were probed with anti-BDNF (1:1000, ab13867; Abcam, Cambridge, UK), anti-proBDNF (1:500, ab203294; Abcam), anti-p75NTR (1:1000, no. 4504; Cell Signaling Technology, Danvers, MA, USA), anti-GAPDH (1:3000, ab8245; Abcam) and anti-β-actin (1:1000, ab8226; Abcam). Data were quantified using Image Studio Lite 5.2 (LI-COR Biosciences, USA).

2.10. RNA extraction and RT-PCR analysis

Periodontal tissue, prefrontal cortex and hippocampal tissue of mice was homogenized, and total RNA was isolated using TRIzol reagent (Thermo Scientific). RNA was reverse transcribed into cDNA with the RT-PCR kit (Takara, Shiga, Japan). RT-PCR analyses were subsequently carried out with SYBR premix EX TaqTM (Takara) according to the manufacturer’s instructions in a Light Cycler 480 System (Roche Diagnostics, Basel, Switzerland). Transcription levels were calculated with the 2−ΔΔCT method. The following primer sequences were used:

Pg, 5′-GGCCTCAACGTTCAGCC-3′(forward) and 5′-CAGGAATTCGCCGTGC-3′(forward); Fn, 5′-GGATTTATGGGCGTAAAGC-3′(forward) and 5′-GGATTCCT

TACAATATCTAGAA-3′(forward); BDNF, 5′-AGGTCGACGACGA CATCAC

T-3′(forward) and 5′-CTTCGTTGGGGCCGACATT-3′(reverse) and GADPH, 5′-CATCGTAAGACCTCTATGCAAC-3′ (forward) and 5′-ATGGGACCCAGGA

TCCAA-3′(reverse).

2.11. Hippocampal injection

p75NTR was synthesized and inserted into a Cre-dependent AAV virus by Hanbio Company (Shanghai, China). Mouse BDNF protein (1 μg per side) (no. PRP100400, Abbkine) and synthetic AAV (1 μL per side) were injected with a stereotaxic instrument (RA-6N, Narishige, Japan) under general anesthesia. Bilateral injection into the DG of the hippocampus at a speed of 0.5 μL/min (stereotaxic coordinates with reference to the bregma: anteroposterior, −1.94 mm; mediolateral, 1.00 mm; dorsoventral, 2.10 mm) was carried out with glass micropipettes, which remained in the injection site for an additional 5 min. Then, we placed the mice on a warm pad until full recovery.

2.12. LPS injection

The Pg-LPS group, TAK group, and Pg-LPS + TAK group received intraperitoneal injections of Pg-LPS (5 mg/kg; InvivoGen, San Diego, CA, USA) and/or TAK-242 (5 mg/kg, 1 h before administration of Pg-LPS; InvivoGen). We treated the control group with the same volume of saline. The Escherichia coli (E. coli) group received intraperitoneal injection of E. coli-LPS (5 mg/kg, L2630; Sigma, USA).

2.13. Antibiotic therapy (ABT)

Mice received a 1:1 mixture of amoxicillin and metronidazole (M3761, Sigma, USA) in drinking water (1 μg antibiotic per g body weight) and applied to the surface of their mandibular molars in 2% carboxymethylcellulose for 14 days. Control mice received sterile water and vehicle only.

2.14. Statistical analysis

Data are shown as the mean ± standard error of the mean (SEM). For two-group comparisons, we utilized two-tailed Student’s t-tests. For multiple-group comparisons, we utilized one-way analysis of variance (ANOVA) followed by Dunnett’s t test for comparison between control and experimental groups or followed by Turkey’s t test for comparison between each group. For data which were not normally distributed, we used nonparametric Kruskal-Wallis one-way ANOVA on ranks with Dunnett’s multiple comparison test. We performed these analyses with SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and PRISM software 5.0 (GraphPad Software, San Diego, California, USA).

3. Results

3.1. Pg-induced depression-like phenotypes in mice

To identify whether pathogenic periodontal bacteria cause depression in mice, we generated a mouse model of periodontitis by orally colonizing 6-week-old wildtype (WT) C57BL/6/J female mice with the known periodontitis-inducing bacteria Pg or Fn (Fig. 1A). We detected the mRNA levels of Pg and Fn in periodontal tissues, which was significantly increased in mice colonized with Pg and Fn respectively (p < 0.05, Fig. 1B, C). We verified the success of our periodontitis model by HE staining (Fig. 1D) and evaluating alveolar bone loss (ABL) in the mandibular bone which was increased in mice colonized with Pg.
A

4 weeks +/- colonization with Pg or Fn

Micro CT, IHC, qPCR, WB, ELISA, RNA-seq

B

![Graph showing relative expression]

C

![Bar chart showing mRNA levels]

D

![Images showing tissue sections]

E

![Images showing dental structures]

F

![Graph showing alveolar bone loss]

G

![Graph showing TST immobility time]

H

![Graph showing FST immobility time]

I

![Graph showing weight over time]

J

![Images showing network structures]

K

![Images showing network structures]

L

![Graph showing total distance and curvature time]

M

![Graph showing total distance and curvature time]

N

![Graph showing time spent in open area]

O

![Graph showing time spent in open area]

(caption on next page)
and Fn (p < 0.01, Fig. 1E, F). Our results showed that both bacteria successfully induced periodontitis.

After four weeks of bacterial treatment, we conducted behavioral tests for depression and anxiety. TST and FST were performed to assess depression-like behaviors. Immobile durations in both tests increased significantly in the Pg- and Fn-treated groups compared with the control group (p < 0.001, Fig. 1G, H). However, no significant changes were identified in the Fn group (p > 0.05, Fig. 1G, H). Furthermore, body weights of the Pg group decreased significantly compared with those of control group at days 20 and 24 (p < 0.05, Fig. 1I). We then performed OFT and EPM to evaluate anxiety. Neither the Pg nor Fn groups showed significant differences in total distances traveled and time spent on the central grid in the OFT (p > 0.05, Fig. 1J, L, M) or in average time in open arms and closed arms in EPM (p > 0.05, Fig. 1K, N, O).

Additionally, ABL was significantly correlated with immobility durations in TST (R^2 = 0.59, p < 0.0001; Fig. 1F) and FST (R^2 = 0.73, p < 0.0001; Fig. 1G) in control and Pg group. Our results indicated that Pg specifically induced a depression-like phenotype in mice, while Fn had no such effect. Therefore, we focused further experiments on Pg. Serum levels of cortisone, a stress-related hormone, were significantly increased in the Pg group, compared with the control group (p < 0.001, Fig. 1R).

To confirm the systemic inflammation state in the Pg group, we examined inflammatory factors. Tumor necrosis factor (TNF)-α and Interleukin (IL)-1α expression were significantly increased in blood serum (p < 0.05, Fig. 1S). Additionally, the levels of TNF-α, IL-6, and IL-1α were significantly increased in the Pg group compared with the control group (p < 0.001, Fig. 1T, U).

It has been reported that Pg was detected in Alzheimer’s patients (Dominy et al., 2019). To test whether Pg localize in the brain of Pg-treated mice, we conducted qPCR for Pg 16S ribosomal RNA in hippocampal tissue. The result showed Pg mRNA levels were increased in mice colonized with Pg (p < 0.05, Fig. 1V). The result of Western blotting showed Pg-LPS increased dramatically in the hippocampal region of brain Pg-mice (Fig. 1W). While mice colonized with Fn showed no significant change in the level of Fn 16S ribosomal RNA compared with control group (p > 0.05, Fig. 1X). These results demonstrate Pg oral colonization can induce depression-like behavior in mice.

3.2. Astrocyte activation during depression induction by periodontitis in mice

The hippocampus and prefrontal cortex are associated with pathological mechanisms of depression. To further investigate the molecular mechanism of depression induced by Pg, mRNA expression profiles were determined in these brain regions using microarray analysis. The results showed that mRNA levels of the astrocyte biomarker GFAP increased in response to Pg (Fig. 2A). To confirm this result, we performed immunofluorescence against GFAP in the hippocampus and prefrontal cortex (Fig. 2B). The results showed that GFAP+ astrocytes in the hippocampus of the Pg group were significantly increased, and the number and length of astrocytic processes were also significantly increased (p < 0.05, Fig. 2C–E). However, GFAP+ astrocytes were not detected in the prefrontal cortex (data not shown). The results of these experiments suggested that astrocytic activity in the hippocampus was increased in Pg-induced depressive mice.

3.3. Expression of mature BDNF was reduced in the hippocampus of Pg-treated mice, and hippocampal injection of BDNF ameliorated depression-like behavior

BDNF plays an important role in the pathogenesis of depression. Astrocytes can regulate BDNF maturation, so we assessed BDNF levels in the brain. RNA-Seq results showed that mRNA levels of BDNF did not change significantly in response to Pg treatment (Fig. 2A). We performed RT-PCR tests to further confirm this result (p > 0.05, Fig. 2B). This result suggested that Pg may regulate BDNF levels through post-transcriptional mechanisms. Next, we measured BDNF protein levels in the hippocampus. Levels of mature BDNF (mBDNF) protein in the Pg group were lower, while levels of proBDNF in the Pg group were higher than those in the control group (Fig. 3C). To further determine whether changes in mBDNF were involved in Pg-induced depression-like symptoms, we injected mBDNF into the DG region of the hippocampus (Fig. 3A, D). We found that mBDNF injection decreased the immobility time of Pg-mice in TST and FST (p < 0.05, Fig. 3E, H) and increased the latency time of Pg-mice in FST (p < 0.05, Fig. 3G). The results showed that mBDNF injection rescued Pg-induced depression-like phenotypes in mice. These data suggested that reduction of mBDNF is a potential mechanism of Pg-induced depression.

3.4. Expression of astrocytic p75NTR was reduced in Pg-treated mice and overexpression of p75NTR in the hippocampus rescued depression-like behaviors in mice

Based on the role of astrocytic p75NTR in BDNF maturation and the observed increase in activation of astrocytes (Fig. 2B–E), we measured expression of p75NTR in astrocytes in Pg-induced depressive-like mice (Fig. 4A). The expression of p75NTR was significantly lower in astrocytes in the Pg group (p < 0.001, Fig. 4B). We also measured p75NTR expression in neurons (Fig. 4C). No significant differences in p75NTR expression were detected in neurons (p > 0.05, Fig. 4D).

We further injected AAV-p75NTR into the hippocampal DG region of hGAFP-Cre mice (Chen et al., 2017; Zhuo et al., 2001) resulting in overexpression of p75NTR in astrocytes (Fig. 4E–G). After four weeks of Pg colonization, immobility durations in TST and FST were lower in the group that received AAV-p75NTR injection compared with the AAV-GFP group (p < 0.05, Fig. 4I, K). This demonstrates that p75NTR overexpression rescues the Pg-induced depression phenotype.

3.5. LPS from Pg intraperitoneal injection induced depression-like behavior, which was ameliorated by pretreatment with the TLR-4 inhibitor TAK242

LPS is an important factor in Pg. We hypothesized that Pg-induced depression may be caused by LPS produced by Pg (Pg-LPS). To test this possibility, Pg-LPS was intraperitoneally injected into mice (Fig. 5A).
LPS injection increased the immobility durations in TST and FST compared with the control group ($p < 0.05$, Fig. 5C, E). The results showed that Pg-LPS also induced depression-like phenotypes in mice (Fig. 5B, C), suggesting Pg-LPS is a potential cause of depression-like behavior in mice treated with Pg.

TLR4 is the major receptor for LPS on astrocytes, and LPS can enter the brain via the brain blood barrier (BBB) to activate TLR signaling (Zhao et al., 2017). The TLR4 inhibitor TAK242 has been used to protect the brain from ischemic injury (Hua et al., 2015). We injected TAK242 into the abdomens of the mice concurrent with Pg-LPS treatment (Fig. 5A). There were no significant changes in latency time or immobility durations in TST or FST ($p > 0.05$, Fig. 5C–F). In the TAK242 + Pg-LPS group, TAK242 injection significantly decreased immobility durations compared with the Pg-LPS group ($p < 0.05$, Fig. 5C, E), which showed that Pg-LPS did not induce a depression-like phenotype in mice after inhibition of TLR4 pathway activity. These results suggest that Pg-LPS induced a depression-like phenotype in mice by activating TLR4-mediated pathways in astrocytes.

Immunostaining for GFAP showed that the number of GFAP$^+$ astrocytes in the hippocampal area of the Pg-LPS group was significantly increased ($p < 0.05$, Fig. 5F, I). Additionally, the number and length of astrocytic processes were also significantly increased ($p < 0.001$, Fig. 5H, I). TAK242 inhibited these increases ($p < 0.05$, Fig. 5G–I).

Next, we determined the protein levels of p75NTR in the hippocampus of Pg-LPS-injected mice and found that p75NTR levels were significantly decreased compared to those of controls (Fig. 5J). Our results suggest that Pg-LPS may downregulate p75NTR protein levels in astrocytes via the TLR4 signaling pathway.

### 3.6. Antibiotic therapy rescued depression-like behavior in Pg-colonized mice

Based on our results, we hypothesized that antibacterial therapy (ABT) of periodontitis could rescue Pg-induced depression-like behaviors. After two weeks of topical and systemic antibacterial drug administration (Fig. 6A), the immobility times of both TST and FST in the Pg + ABT group were significantly decreased compared with the Pg group without antibiotic treatment ($p < 0.05$, Fig. 6C,E). The latency to immobility of both TST and FST in the Pg + ABT group significantly increased compared with the Pg group without antibiotic treatment ($p < 0.05$, Fig. 6B, D).

Altogether, we hypothesize that Pg activates the TLR4 signaling pathways in astrocytes in the hippocampal region, resulting in down-regulation of p75NTR expression, leading to inhibition of BDNF maturation. Lack of mature BDNF results in neuronal dysfunction and subsequent depression-like phenotypes in mice (Fig. 6F).

### 4. Discussion

In this study, we demonstrated that the periodontal bacteria Pg induces depression-like behavior in mice. The underlying mechanism maybe increase activation of astrocytes via Pg-LPS/TLR4 signaling pathway, resulting in downregulation of astrocytic p75NTR and inhibition of BDNF maturation. Pg was applied to the molars of mice every other day for 4 weeks, and the alveolar bone loss was measured to confirm successful initiation of Pg-induced periodontitis. Detection of Pg 16S ribosomal RNA and increase in inflammatory response in periodontal tissue indicated that Pg successfully colonized periodontal tissues. The Pg-treated mice had...
increased immobility duration on FST and TST. Additionally, other related parameters were tested including body weight, inflammatory factors, and cortisone levels. These results suggest that *Pg* is capable to induce depression-like phenotype in mice. We also tested another pathogenic bacteria of periodontitis—*Fn*. Although *Fn* also induced periodontitis, it did not induce depression-like behaviors in our experiments. This result suggested that *Pg* is a specific periodontitis pathogen that induces depression-like symptoms in mice.

Astrocytes play an important role in regulating neuronal structure and function (Verkhratsky and Nedergaard, 2018). Changes in GFAP levels and astrocytic morphology indicate alternation of astrocytic activity (Rajkowska and Stockmeier, 2013). Previous reports are conflicting with regard to expression levels of GFAP in postmortem studies of depression (Miguel-Hidalgo et al., 2000). Here, our results showed that oral colonization of *Pg* increased GFAP expression in the hippocampus. We postulate that bacteria or inflammatory factors activate astrocytes, and social stress may cause the opposite effect. It would be interested in exploring the astrocyte role in the two different causes for depression in future work. Additionally, previous reports showed that the number and activation of microglia were increased in patients with depression (Tay et al., 2017). However, we did not find significant changes in the mRNA of microglia markers Iba1 and CX3CR1 in the present study (data not shown). This is due to the relatively low-doses of *Pg*/*Pg*-LPS used in our study, which were not sufficient to activate microglia in the brain, consistent with previous report about rats treated with low-dose LPS (Galic et al., 2008). Thus, we did not evaluate microglia further in our study. However, changes to structure and/or function of microglia cannot be ruled out. Additionally, both the mPFC and hippocampus are involved in depression. We found that hippocampal astrocytes, but not mPFC astrocytes, were activated, consistent with previous studies (Marathe et al., 2018). This difference may have resulted from the incomplete BBB in the hippocampus (Obermeier et al., 2013), which may allow greater permeation of LPS and inflammatory factors into the hippocampus, resulting in greater activation of LPS/TLR-4 signaling.

BDNF regulates synaptic plasticity and promotes neuronal regeneration (Sasi et al., 2017). Previous studies have shown that levels of BDNF decrease and levels of proBDNF increase in depression (Bai et al., 2016). However, the underlying mechanisms have not been characterized. Astrocytes can modulate neuronal activity and synaptic

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**Fig. 2.** Astrocyte activation during depression induced by *Pg* in mice. (A) RNA-sequencing of prefrontal cortex and hippocampus. BDNF and GFAP are highlighted with the red box. Peri c indicates cortex of *Pg*-induced periodontal group. Peri h indicates hippocampus of *Pg*-induced periodontal group. Con c indicates cortex of control group. Peri h indicates hippocampus of control group. (B) Representative images of GFAP+ astrocytes in the hippocampus of the control group and *Pg* group (scale bar = 20 μm). (C) Number of GFAP+ astrocytes, (D) total branch numbers and (E) total branch length of GFAP+ astrocytes in the hippocampus (n = 15 per cohort). *p < 0.05, **p < 0.001 compared to the control group.
density via production of neurotrophin, or transformation of premature neurotrophin to mature neurotrophin via the membrane receptor p75NTR (Bergami et al., 2008). Interestingly, we found that p75NTR levels were dramatically decreased in activated astrocytes in our depressed mice. To test whether BDNF and p75NTR were involved in Pg-induced depression, we infused BDNF and overexpressed astrocytic p75NTR into the hippocampus. Both reversed Pg-induced depression-like behaviors in mice. Thus, our results suggest that p75NTR might mitigate depression and prompt BDNF maturation. In addition, to detect whether other Gram-negative bacteria-LPS works in the same way, we examine the change of mice behavior and the brain tissue following intraperitoneal injection of E. coli-LPS in mice (see Supplementary Fig. S1). The results showed that E. coli-LPS also induced depression-like behavior after E. coli-LPS treatment, but we did not find the change of astrocyte activation and p75NTR level. It indicates that Pg functions in a different way, compared with E. coli. It is still unclear this reason is because that the structure difference between E. coli-LPS and Pg-LPS makes them activating different signal way or E. coli-LPS fail to penetrate into brain.

In the central nervous system (CNS), TLR-4 is predominantly expressed on microglia (Zhao et al., 2014). TLR-4 dysfunction has been implicated in various CNS neuropathological conditions including depression, Alzheimer’s disease, and Parkinson’s disease (Wu et al., 2015). Previous reports have shown that neuroinflammation is involved in those pathogenic mechanisms (Liu et al., 2014). However, the function of TLR-4 in astrocytes remains elusive. Our results suggest that astrocytic TLR-4 plays a role in the pathogenesis of depression. We will examine changes in TLR-4 activity in astrocytes in future work. Additionally, we first found that TLR-4 activation downregulates the protein level of p75NTR in astrocyte, but the underlying mechanism is still unclear. We will identify the molecular mechanism in future work.

Pg contains a variety of virulence factors including LPS, flagella, and toxic proteases (Xie, 2015). Previous reports have shown that LPS can dramatically increase astrocyte activation (Shen et al., 2016), and greater levels of LPS were positively correlated with Alzheimer’s disease and epilepsy (Teixeira et al., 2017). We found the mice injected with Pg-LPS also showed depression-like behaviors, which could be blocked by TAK242, which is the inhibitor of the LPS receptor TLR-4. Our results indicate that Pg-LPS is an important virulence factor that induce depression in mice. Further, another virulence of Pg, Pg-gingipain, has also been identified in the brain and as involved in the pathologic process of Alzheimer’s disease (Dominy et al., 2019). However, whether Pg-gingipain is involved in pathology of depression is still unknown. We will test the role of Pg-gingipain in the pathogenesis of Pg-induced
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Fig. 4. p75NTR in astrocytes decreased in Pg-treated mice, and overexpression of p75NTR in the hippocampus rescued depression-like behavior. (A) Representative images of GFAP + astrocytes and p75NTR in the hippocampus of the control and Pg group (scale bar = 20 μm). Arrow indicates local magnification. (B) Relative mean density of p75NTR in GFAP + astrocytes (n = 50 per cohort). (C) Representative images of neurons and p75NTR in the hippocampus of the control and Pg group (scale bar = 50 μm). Arrow indicates local magnification. White arrowhead indicates p75NTR expression in neurons. (D) Relative mean density of p75NTR in neurons (n = 50 per cohort). (E) Schematics of AAV vectors engineered to overexpress a GFP control construct or p75NTR. (F) Schematic of experimental designs. (G) Illustration of bilateral viral injection of AAV-p75NTR in DG of hippocampus. (H) Latency to immobility and (I) immobility time in TST. (J) Latency to immobility and (K) immobility time in FST (Control group: n = 7, Pg + AAV-GFP group: n = 7, Pg + AAV-p75NTR group: n = 9). *p < 0.05, ****p < 0.0001 compared to the control group. #p < 0.05 and ###p < 0.001 compared to the Pg + AAV-GFP group.

Fig. 5. Intraperitoneal injection of LPS produced by Pg induced depression-like behavior, which was ameliorated by pretreatment with the TLR-4 inhibitor TAK242. (A) Schematic of experimental designs. (B) Latency to immobility and (C) immobility time in TST. (D) Latency to immobility and (E) immobility time in FST (Control group: n = 8, Pg-LPS group: n = 8, TAK242 group: n = 8, TAK242 + Pg-LPS group: n = 8). (F) Representative images of GFAP + astrocytes in the hippocampus of the control, TAK242, Pg-LPS and TAK242 + Pg-LPS groups (scale bar = 20 μm). (G) Number GFAP + astrocytes. (H) Total branch numbers and (I) total branch length of GFAP + astrocytes in the hippocampus (n = 10–13 per cohort). (J) Western blot analysis of p75NTR in the hippocampus of Pg-LPS-treated mice. *p < 0.05 and ***p < 0.001 compared to the control group, #p < 0.05 and ###p < 0.001 compared to the Pg-LPS group.
In the present study, we provided mice with *Pg*-induced periodontitis antibiotics for two weeks. Interestingly, we found that this treatment rescued the depression phenotypes in these mice. Selective serotonin reuptake inhibitors are an effective strategy for treating depression, but some patients have serious side effects or fail to respond well (Kessler et al., 2005). Our study demonstrates a potential novel strategy for the clinical treatment of depression in humans.

In conclusion, our work demonstrates that *Pg* plays a role in the pathogenesis of depression in mice. The underlying mechanism is that *Pg* activates astrocytes in the hippocampus through binding TLR4 with *Pg*-LPS, downregulates p75NTR on astrocytes, and impairs BDNF maturation, resulting in depression. *p* < 0.05, ***p* < 0.001 and ****p* < 0.0001 compared with the control group. #p* < 0.05, and ###p* < 0.001 compared with the *Pg* group.

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**Author contributions**

YX Wang conducted the experiments and prepared the figures. XN Kang, Y Cao, DX Zheng, YM Lu, CF Pang and Z Wang contributed to the behavioral experiments and statistical analysis. Y Peng and B Cheng supervised the project and wrote the manuscript.
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