RE: Revision of manuscript number JAD 14-0315R1

Professor George Perry (Editor-in-Chief) **May 15, 2014**
Journal of Alzheimer's disease
College of Sciences
University of Texas at San Antonio
One UTSA Circle
San Antonio, Texas 78249
USA

Dear Professor Perry:

RE: Revision of manuscript number JAD 14-0315R1

The authors - Sophie Poole (PhD Student), Sim K. Singhrao (Snr Res fellow), Sasanka Chukkapalli (PDRA), Mercedes Rivera (Research staff), Irina Velsko (PhD Student), Lakshmyya Kesavalu (Associate Professor of Periodontology) and StJohn Crean (Professor of Medicine in Dentistry) - wish to resubmit their re-revised manuscript **JAD 14-0315R1, Active invasion of *Porphyromonas gingivalis* and infection-induced complement activation in ApoE$^{-/-}$ mice brains.**

Editorial comments:

We thank Professor Perry and the editorial staff for their patience and support for the opportunity to re-revise this manuscript. To the best of our knowledge, the manuscript is prepared according to instructions to authors and in American English. As this is a re-revision, we have accepted the changes from the primary revision and tracked new changes to the current manuscript as per reviewers' comments.

Reviewer 1

Rev 1: suggested "It appears that the authors rushed to respond to the reviewers' critics instead of improving the manuscript based on the reviewers' critics".

Our response: We agree with the reviewer comment and we now have addressed the helpful comments.

Rev 1: suggested "In its form the manuscript is difficult to read and follow. The entire manuscript suffers from lack of clarity due to long, vague sentences. The manuscript should be re-written with great attention to language and message.

Our response: We apologize to the reviewer for the difficulty in reading and following the article's previous version. We took more care this time and have done major revision, in accordance with all of the reviewers' suggestions. Consequently, some references have been eliminated and the manuscript should reflect these changes to a publication standard in its present form.

Rev 1: suggested that there was no link between F. nucleatum infection and its association with human brain abscess formation as reflected by reference number [39].

Our response: We apologize to the reviewer for the confusion with regard to the reference. We agree with the reviewers' view and felt that it was better to delete the human abscess connection with the infection used in the study as it contributed little to the overall message. Therefore reference # 39 as quoted in the reviewer comments above has been deleted in this revised version.

Rev 1: said another bad example was: "This study explored the hypothesis that infectious agents and /or their components from oral diseases such as periodontitis can access the brain and modulate local CNS inflammation and thereby represent a component of AD pathology. …..What represents a component of AD? Long sentence and difficult to follow.

Our response: We agree; this important information was poorly explained. We have now made every attempt to correct the English and clarify the inflammatory component of AD.

Rev 1: Said "What early pathological lesions? Not well defined"?

Our response: The major re-revision of the manuscript is more focused and this confusing information is deleted and explained in a more clear and concise manner.

Rev 1: Said Methods: lacked "A brief paragraph describing the general aspect of the procedures, specific measurements(Dependent variables) and assays done to measure the dependent variables would bring greater clarifications" as per original reviewer's comments. When the readers are reading this paragraph they should know exactly what experimental procedures were done, timing,what tissues were collected, what was measured and how. And why?

Our response: We agree with this comment and have made extensive changes in the methods section.

Rev 1: Said In methodology section no details about mono-infections. For example:describe the experimental assignment for the infections, then describe mono, then poli-infection and then state the antibiotics.

Our response: We have modified the methods section as suggested by the reviewer in this revised version.

Rev 1: Said On page 17, the authors stated: In addition, a modified methenamine silver impregnation technique as previously described by Cole et al.,[46] was used to demonstrate both the Aplaques and NFT's. All sections were also stained with 1% aqueous thioflavin T for fibrillar amyloid. I searched the document for these results and could not find them.

Our response: We agree with the reviewer. It was the corresponding authors' oversight as she was instrumental in developing this technique originally for the Cole et al. article.

However, the author also applied the technique to resin embedded AD brain tissue and that article by Singhrao et al. is in the public domain. The Cole et al. reference has been replaced by Singhrao et al.

Rev 1: Said the authors deleted this section: A semi-quantitation approach was taken by manually counting the number of cells/area for all brains in each infected group and compared with the sham group to assess gliosis. I cannot imagine that any reviewer would object to a methodology describing quantification.

Our response: We have re-instated the statement as suggested by the reviewer.

Rev 1: Said "In the results section, the authors stated: PCR analysis revealed that none of the three pathogens were detected in any of the brains from sham, mono, and poly-infected groups at both time points (Fig. 1,panels a, b and c). And then: Molecular identity of the organism following cloning of the purified PCR product and direct sequencing using bacterial gene specific primer sets confirmed that it was P. gingivalis FDC381". Could you explain?

Our response: This point has been explained in the appropriate sections of the manuscript.

Rev 1: Said "The discussion is equally difficult to follow". In conclusion, I recommend the re-writing of the manuscript based on the reviewers' critics. Please, analyze and understand the critics and then incorporate them in "your story".

Our response: We have conducted a major revision of the manuscript, incorporating all of the reviewers' comments as suggested to improve the final quality to a publication standard.

We eagerly await your reply.

Yours sincerely,

Sim K. Singhrao

Associate Editor JAD and Corresponding author

Title: Active invasion of *Porphyromonas gingivalis* and infection-induced complement activation in ApoE$^{-/-}$ mice brains

Authors: Sophie Poole[1°], Sim K. Singhrao[1*], Sasanka Chukkapalli[2 °], Mercedes Rivera[2], Irina Velsko[2], Lakshmyya Kesavalu[2,3•], StJohn Crean[1•]

[*1]Oral & Dental Sciences Research Group,

School of Medicine and Dentistry,

University of Central Lancashire,

Preston, PR1 2HE

U.K.

[2]Department of Periodontology,

[3]Department of Oral Biology,

College of Dentistry, University of Florida,

Gainesville, Florida 32610-0434

USA.

[°] contributed equally to the model (in USA) and laboratory–based analyses of the brain (UK)

[•]Contributed equally

Running Title: Oral pathogen in brain

***Corresponding author:**

Dr S. K. Singhrao

Tel: +44 (0) 1772 895137

Fax: +44 (0) 1772 892965

E-mail: SKSinghrao@uclan.ac.uk

Abstract

Periodontal disease is a polymicrobial inflammatory disease that leads to chronic systemic inflammation and direct infiltration of bacteria/bacterial components, which may contribute to the development of Alzheimer's disease. ApoE$^{-/-}$ mice were orally infected (N = 12) with *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia* and *Fusobacterium nucleatum* as mono- and polymicrobial infections. ApoE$^{-/-}$ mice were sacrificed following 12 and 24 weeks of chronic infection. Bacterial genomic DNA was isolated from all brain tissues except for the *F. nucleatum* mono-infected group. Polymerase chain reaction was performed using universal 16s rDNA primers and species--specific primer sets for each organism to determine whether the infecting pathogens accessed the brain. Sequencing amplification products confirmed the invasion of bacteria into the brain during infection. The innate immune responses were detected using antibodies against complement activation products of C3 convertase stage and the membrane attack complex. Molecular methods demonstrated that 6 out of 12 ApoE$^{-/-}$ mice brains contained *P. gingivalis* genomic DNA at 12 weeks (P = 0.006), and 9 out of 12 at 24 weeks of infection (P = 0.0001). Microglia in both infected and control groups demonstrated strong intracellular labeling with C3 and C9, due to on-going biosynthesis. The pyramidal neurons of the hippocampus in 4 out of 12 infected mice brains demonstrated characteristic opsonization with C3 activation fragments (P = 0.032). These results show that the oral pathogen *P. gingivalis* was able to access the ApoE$^{-/-}$ mice brain and thereby contributed to complement activation with bystander neuronal injury.

Key words: Alzheimer's disease, chronic periodontitis, periodontal bacteria, inflammation,

Introduction

Alzheimer's disease (AD) is a form of dementia associated with cognitive decline and irreversible memory loss. The pathological hallmarks of AD brains are an accumulation of intracellular hyper-phosphorylated tau-positive neurofibrillary tangles (NFT) together with insoluble, fibrillary amyloid (Aβ4) plaques, which are traditionally recognized as being triggers that stimulate glial cell activation and initiate local innate immune responses [1]. AD has a complex aetiology in which the genetic makeup of the individual and environmental factors play a role. The late-onset form of AD is particularly interesting as its aetiology remains unknown despite the known genetic risk factors, including apolipoprotein E (ApoE) gene and its E4 allele inheritance [2, 3]. This risk factor is associated with severe AD pathology and an enhanced inflammatory response by microglia [4].

Peripheral infections also serve as a significant risk factor affecting mental health as demonstrated in clinical studies in which cognitive decline and deteriorating memory are reported [5-7]. A range of infective agents is consistently being linked to AD [8], including viruses such as the Herpes simplex virus type 1 (HSV-1) [9]; bacteria such as *Chlamydophila pneumoniae* (*C. pneumoniae*) [10]; and various types of spirochetes, including *Borrelia burgdorferi* (*B. burgdorferi*) [11-13] and periodontal *Treponema* spp., [14] and more recently *Porphyromonas gingivalis* (*P. gingivalis*) [15]. *P. gingivalis* and some oral *Treponema* species are invasive and virulent within their original niche where they induce gingival inflammation that leads to connective tissue degradation and alveolar bone resorption around teeth [16, 17]. Once the junctional epithelium that links the gingiva to the tooth enamel transforms to pocket epithelium, pathogenic bacteria induce bacteremia and initiate systemic inflammation by infiltrating the local blood vessels [18-20]. These factors may lead to various chronic inflammatory disorders such as cardiovascular disease(s) [21,

22], diabetes [23], rheumatoid arthritis [24-26], premature births [27], and AD [14, 15, 28, 29].

Clinical studies by Stein et al. [28] support a strong association between tooth loss due to periodontal disease and the development of AD. They noted a greater rate of cognitive decline occurring in carriers of the Apolipoprotein E4 (ε4 allele) variant with fewer teeth [30]. Although chronic infection by *Treponema pallidum* is widely accepted for the atrophic form of general paresis, it and *B. burgdorferi* infections (etiological bacteria for Lyme disease) are also reported to result in dementia [11-13]. These spirochete infections give rise to the similar pathological hallmark features such as Aβ4 plaques and NFTs seen in AD [11-13]. This is regarded as a direct link between spirochete infections and the development of AD. *C. pneumoniae* and HSV-1 infections of the brain also appear to be associated with the Aβ4 deposition observed in AD [9, 10, 12]; however, their role as infection by individual pathogen or occurring as co-infections with the invading spirochetes remains under investigation [12]. *T. denticola* and *P. gingivalis* oral infections of the brain are also reported [14, 15], but their direct involvement with the deposition of Aβ4 and NFTs is not clear.

Inflammation in the brain is characterized by the presence of reactive microgliosis and astrocytosis (inflammatory phenotype) and is an accepted component of AD pathology [1]. Traditionally, the inflammatory component of the pathology in AD is believed to be the result of cytokines, oxidative stress, and complement activation, including the membrane attack complex due to the hallmark proteins of AD [1]. However, the fact that pathogens are implicated in some forms of central nervous system (CNS) diseases that result in the eventual development of AD [11-13], suggests that the existing hypothesis cannot exclude a possible role of chronic infections generating an inflammatory pathology in AD. Concerning chronic infections in AD brains, in 2008 two independent research groups implicated the indirect role of periodontal pathogens and/or their virulence factors in the development of AD [31, 32]

involving acute- -phase proteins, including cytokines, as a plausible link between periodontal bacteria and inflammatory AD pathology. Miklossy [2008], proposed a direct link between oral spirochetes and AD via bacterial infection of the brain in which either the spirochetes or their virulence factors activate the classical and the alternative pathways of complement, resulting in vital cell loss via the membrane attack complex [1333]. Thus, the presence of cytokines and/or an activated complement cascade can be used as a marker to measure CNS inflammation in this context. Local inflammation and complement activation induced by direct, persisting infection of the brain by various types of spirochetes is also implicated as reviewed elsewhere [33]. Researchers have also

Further demonstration of a high titer of antibodies against periodontal pathogens in the serum of elderly who progressed to AD also suggests the possible association between periodontal disease and AD [34].

Poor oral hygiene [35] is strongly linked to the development of dementia; however to date there are very few reports establishing an experimental link between periodontal disease and AD. Two studies using human brain tissue explored the impact of periodontal infections on AD [14, 15]. These studies examined AD brain tissue specimens using molecular profiling methodologies to identify seven *Treponema* species [14], and the immunogenic endotoxin, lipopolysaccharide (LPS), from *P. gingivalis* [15].

Focal dissemination of periodontal pathogens from the oral cavity to distant organ sites has long been hypothesized, but few studies have explored this theory. Previous studies using wild-type mice (C57BL/6J) explored the dissemination of periodontal pathogens in an endodontic infection model [36]. However, the study detailed here was unable to trace the dissemination of periodontal pathogens to distant organ sites due to the disadvantages associated with using a wild-type mouse model [36]. The ApoE$^{-/-}$ mouse model, which is a proatherogenic model for co-morbidity studies, is unable to deposit Aβ4 in the brain as the

essential ApoE isoforms are lacking [37]. This mouse serves as a suitable model with which to study the association between periodontal disease and AD as it avoids confounding factors that may result from an overlap of signaling in response to AD hallmark proteins and pathogen-associated molecular patterns. Thus, keeping in view the lack of *in vivo* experimental evidence for a link between periodontal pathogens/disease and AD, the present study aimed to explore such an association using the ApoE$^{-/-}$ mouse as a model. This study also tested the hypothesis that infectious agents and/or their components from oral diseases such as periodontitis can access the brain and modulate local CNS inflammation. To this end, we investigated the role of the oral pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* in accessing the brain of ApoE$^{-/-}$ mice following chronic experimental periodontitis and in contributing to the development of local inflammation as an early pathological lesion in relation to AD.

The present study explored the possibility of specific oral pathogens altering normal functioning of the brain in experimental animals with established periodontitis. In this infection model *F. nucleatum* was used as a bridging organism that co-aggregates with major periodontal bacteria in both supra- and subgingival biofilm development and for the subsequent progression of periodontitis [3~~8~~6-40~~38~~]. ~~Furthermore, *F. nucleatum* has been associated with human brain abscess formation [39].~~

~~One prior study examined the dissemination of the three main periodontal disease-causing organisms *P. gingivalis, Treponema denticola* (*T. denticola*) and *Tanerella forsythia* (*T. forsythia*) as mono- and polymicrobial infections in wild type mice (C57BL/6J) in an endodontic infection [40]. However, this study revealed that none of the pathogens accessed "distant organ sites" hence, the wild type mice did not make a good animal model to explore the focal infection theory [40]. Currently there is no, *in vivo* experimental evidence for a link between periodontal pathogens/disease and AD. This study explored the hypothesis that~~

~~infectious agents and /or their components from oral diseases such as periodontitis can access the brain and modulate local CNS inflammation and thereby represent a component of AD~~

Materials and Methods

Mice, oral infection, and brain

The study involved oral infection of ApoE$^{-/-}$ mice with periodontal pathogens either as mono- or polybacterial for a chronic infection period of 24 weeks. Following the infection period the mice were euthanized and the brain tissue was collected and preserved. Later, using molecular, immunological, and pathological detection techniques we evaluated the invasion of periodontal bacteria into the mice brains.

Microbial strains

P. gingivalis FDC 381, *T. denticola* ATCC 35404, *T. forsythia* ATCC 43037, and *F. nucleatum* ATCC 49256 were used in the study and were routinely cultured anaerobically at 37°C as described previously [41].~~(strain B6.129P2-Apoe$^{tm1Unc/J}$, Jackson Laboratories, Bar Harbor, MA, USA) were purchased and at 8 weeks of age they were randomly assigned to sham-infected, mono-infected (*P. gingivalis*, *T. denticola*, *T. forsythia*,*F. nucleatum*) and polymicrobial-infected groups. Antibiotic treatment (500 µg/mL kanamycin) was administered once for three days followed by the same period in which antibiotic-free water was supplied prior to initiating the first oral lavage with the periodontal bacteria [42]. The aim was to aid adherence of periodontal bacteria by suppressing the murine indigenous oral microflora population. For polymicrobial~~

ApoE$^{-/-}$ Mice oral infection

Eight-week-old male ApoE$^{-/-}$ mice strain B6.129P2-Apoe$^{tm1Unc/J}$ (Jackson Laboratories, Bar Harbor, ME, USA) were randomly assigned to sham-infected, mono-infected (*P. gingivalis*,

T. denticola, *T. forsythia*,*F. nucleatum*) and polymicrobial-infected groups, (N = 12 in each group). This mouse study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, USA. All procedures were performed in accordance with the approved protocol guidelines (Protocol # 201004367) set forth by the Institutional Animal Care and Use Committee of the University of Florida. The University of Florida has an Assurance with the Office of Laboratory Animal Welfare and follows Public Health Service policy, the Animal Welfare Act and Animal Welfare Regulations, and the *Guide for the Care and Use of Laboratory Animals*, USA. ApoE$^{-/-}$ mice were administered with 500 µg/mL kanamycin in drinking water for 3 days followed by a mouth rinse with 0.12% chlorhexidine gluconate [42] before the first oral lavage with the periodontal bacteria [42] to suppress the murine indigenous oral microflora. While mono-infections involved a bacterial inoculum of 10^9 cells/mL of respective bacteria, the polymicrobial-infection constituted an inoculum of 5×10^9 combined bacteria/mL, ~~*P. gingivalis* was mixed with an equal quantity of *T. denticola* for 5 min; subsequently, *T. forsythia* was added to the culture tubes containing *P. gingivalis* and *T. denticola*, and cells were mixed thoroughly and allowed to interact for an additional 5 min. *P. gingivalis*, *T. denticola*, and *T. forsythia* were mixed and added to *F. nucleatum* with an equal volume of 4% (w/v) sterile carboxymethylcellulose (CMC; Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS). This mixture was used for oral infection (5×10^9 bacteria/mL) in ApoE$^{-/-}$ mice~~ as described previously [41, 42]. This investigation is part of an on-going collaboration with the University of Florida and the University of Central Lancashire (UCLan) (MTA Ref. No. A10415). Ethical approval was obtained from the Animal Projects Committee of UCL~~l~~an for research on animal tissues as secondary users (Ref. No. RE/11/01/SS). ~~as well as in accordance with the approved protocol guidelines~~

~~(Protocol # 201004367) set forth by the Institutional Animal Care and Use Committee of the University of Florida.~~

Collection and storage of brain tissue specimens

The mouse brains were removed following 12 and 24 weeks of oral infection as well as sham-infection and separated into two halves. One cerebral hemisphere was immediately stored at -80°C in RNA*later*® buffer for subsequent molecular biology analysis and the other half fixed in 10% neutral buffered formalin for histopathological analysis.

Genomic DNA Isolation

To confirm the spread of periodontal pathogens from the mouth to the brain of ApoE$^{-/-}$ male mice, genomic DNA was isolated from the brains of all the infected and sham-infected groups. Briefly, frozen brain tissue (25 mg) was removed, close to the circumventricular organs in a bench top microflow cabinet (Astec Microflow Ltd., UK), using the aseptic technique [15]. Following the manufacturer's protocol (Qiagen DNA easy blood & tissue kit 69504), brain tissue was lysed and genomic DNA was isolated manually using ethanol precipitation.

DNA Amplification and sequencing

Polymerase chain reaction (PCR) was performed using a thermocycler (Veriti, Applied Biosystems, UK), initially using the universal bacterial primers (Table 1a) from the 16s rDNA bacterial genes [43]. For the bacterial-specific gene amplification, the primer sets from Figuero et al.~~,~~ [44] and Rivera et al.~~,~~ [415]~~,~~ (Table 1b) were employed, adhering to the published PCR protocols [41, 44~~, 45~~]. ~~The negative controls contained all PCR reagents except for the sample DNA.~~ PCR products were analyzed using agarose gel electrophoresis (1.5 %) and visualized in the Gene Genius bio-imaging system, and images were captured using the Gene snap software (Syngene, UK). The PCR product was cleaned in MicroCLEAN DNA Cleanup® reagent (Web Scientific Ltd.,) and cloned using the TA

TOPO cloning kit (Invitrogen) according to the manufacturer's instructions. Following successful colony screening, a mini culture (10 ml) of each of the selected colonies was set up overnight and plasmid DNA isolated using a Qiaquick kit (Qiagen). This was followed by sequencing (40 ng) with the M13 forward or reverse primers (TA TOPO cloning kit, Invitrogen) and using the BigDye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The sequencing parameters were an initial denaturation step at 96°C for 1 min and 25 cycles involving (96°C for 10 sec), annealing (50°C for 5 sec), and elongation (60°C for 4 min) according to Paster et al., [43]. Following sequencing the results were submitted to BLAST nucleotide search engine for 16s DNA genes (http://blast.ncbi.nlm.nih.gov/) to identify the organism(s) with 99-100% match with at least 200 bases.

Immunodetection of periodontal pathogens in mouse brain tissue

Isolation of total protein from mouse brain tissue

In each case a 3-mm-thick section of the cortical brain was minced in the lysis buffer containing protease inhibitors [15]. The total protein concentration of all cell lysates was determined as described previously [15]. A number of positive and negative controls were kindly provided as gift reagents and their sources are identified in Table 2. These were sterile bacterial growth medium (medium control) and *P. gingivalis* culture supernatant as described in Poole et al., [15], purified recombinant *T. denticola* protein (FhbB) [45], and ready-to-use *T. forsythia* whole cell lysate [46].

~~*Mouse brain tissue cell lysate*~~

~~A 3 mm thick section of the cortical brain was minced in the lysis buffer containing protease inhibitors [15]. Total protein concentration of all cell lysates was determined as described previously [15].~~

Immunoblot analysis

Immunoblotting was performed under reducing conditions in which up to 60 µg per lane of total protein from all brain specimens was loaded [15] on SDS-PAGE gels of variable percentages (7.5% gels were used for high--molecular--weight proteins such as the S-layer of *T. forsythia*, 12.5% for gingipains and LPS from *P. gingivalis* and 15% w/v gels were used for the low--molecular--weight proteins detected by anti-*T. denticola* antibodies). Following electrophoresis, proteins were electro-transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P; Millipore, UK). The membranes were blotted with mouse anti-*P. gingivalis* (clone 1B5), rabbit anti-*T. forsythia* against the S-layer, and anti-*T. denticola* ATCC 35405 antibody against FhbB protein generated in rats (sources of antibodies and their dilutions used are listed in Table 2).

Histopathological staining of brain tissue

The formalin-fixed brain tissue was thoroughly washed in PBS and the intact hemisphere was divided into the frontal cortex, temporal lobe inclusive of the hippocampus, and the brain stem and cerebellum. The specimens were then processed and embedded in paraffin wax. The tissue blocks with temporal lobe inclusive of the hippocampus were sectioned (5 µm in thickness) using the Leica RM2235 microtome.

Cryo-sections (10 µm thickness) from frozen unfixed brain tissue (hippocampus) were cut using the Leica CM1850 cryostat (Leica UK). Both paraffin wax and cryo-sections were collected onto superfrost+® glass slides (Leica UK). The cryo-sections were either used immediately or stored at -80°C until required for further use. Rehydrated paraffin wax sections were examined for morphology following staining with Haematoxylin and Eosin (H/E). In addition, a modified methenamine silver (silver impregnation) technique adapted from resin-embedded-tissue specimens as previously described by Singhrao~~Cole~~ et al.~~,~~ [4~~6~~] was used to demonstrate ~~both~~ the Aβ4 plaques and the NFTs. All sections were also stained

with 1% aqueous thioflavin T as a standard neuropathology technique for detecting fibrillar amyloid deposition.

Immunofluorescence labeling of periodontal pathogens in brain tissue

Antigen retrieval was carried out on rehydrated paraffin wax sections for labeling with goat anti-Iba1 (Abcam) by microwave heating of tissue sections, at 750 W power for 35 min in 10 mM citric acid buffer (pH 6.0). The infected as well as sham-infected control brain sections were incubated in primary antibodies and subsequently in secondary detection antibodies. Rehydrated paraffin wax sections were immunolabeled with rabbit anti-glial fibrillary acidic protein (GFAP) (Table 2) and the calcium binding protein marker Iba 1 (AbCam). For formalin fixative sensitive antibodies, tissue sections from frozen brains were stabilized by fixation in cold acetone for 10 min followed by a 5 min wash in PBS. Tissue-associated endogenous fluorescence was quenched for 10 min in 50 mM glycine/PBS. All brain tissue specimens were immunolabeled using the mouse anti-*P. gingivalis* (1B5), anti-*T. denticola* ~~(ATCC 35405 antibody~~ against FhbB protein~~),~~ and anti-*T. forsythia* (against S-layer) and for complement C3 activation products rat anti-C3b/iC3b/C3d (Hycult Biotech), and a rabbit anti-C9 neoepitope to detect the membrane attack complex. The dilutions for incubation of sections in primary antibodies are given in Table 2. Where appropriate, the antibodies were diluted in block solution containing 0.01% normal serum (goat serum for GFAP, *P. gingivalis* (1B5), *T. denticola* (FhbB), *T. forsythia* (S-layer), C3b/iC3b/C3d and C9 neoepitope; rabbit serum for Iba 1) in PBS pH 7.3 and 0.25% tween 20. FITC-conjugated secondary detection antibodies were goat anti-rabbit (Sigma-Aldrich Ltd., UK) diluted 1/200 and rabbit anti-goat Alexa Fluor 488® and goat anti-rat Alexa Fluor® 488 (Molecular Probes, UK) diluted 1/1000, in block solution. Sections were mounted under a glass coverslip using the Vectashield® PI (propidium iodide) mounting medium (Vector laboratories, Perterborough, UK). Labeling was observed and images were captured using a 510 series

Zeiss confocal microscope (Carl Zeiss Ltd). A semi-quantitative approach was taken by manually counting the number of cells/area for all brains in each infected group and compared with the sham group to assess glial cell activation.

Statistical analysis

Data are presented as mean ± standard deviation (n≥3 replicates per treatment) and tested for normality and equal variance prior to analysis. Where treatment groups did not meet the assumptions for parametric analysis, the non-parametric Mann Whitney-U test was performed comparing the number of positive cases in each group of infected mice with those in the sham-infected group. Differences were considered significant at $P \leq 0.05$.

Results

Molecular identification of pathogens in brain specimens

Molecular analysis using universal primers failed to detect *T. denticola* or *T. forsythia* in the brain tissues from sham-, mono-, and polymicrobial-infected groups at both time intervals (Fig. 1, Panels a~~-, b and~~ c). The species-specific bacterial gene primers revealed 6 out of 12 ApoE$^{-/-}$ mice brain specimens containing *P. gingivalis* genomic DNA at 12 weeks (Fig. 1 Panel d), which further increased to 9 out of 12 at 24 weeks (Fig. 1 Panel e). These results are highly significant when analyzed by the non-parametric Mann Whitney-U test; P values = 0.006 at 12 weeks and 0.0001 at 24 weeks. The molecular identity of the organism was further confirmed following purification of the amplification product and direct sequencing. A nucleotide basic local alignment search tool (BLAST) identified a 99-100% match with > 200 bases of the submitted sequence for *P. gingivalis*. ~~using bacterial gene specific primer sets confirmed that it was *P. gingivalis* FDC381. Molecular profiling of mono-bacterial infected mice brain specimens did not show the presence of genomic DNA for *T. denticola* and *T. forsythia* (data not shown).~~ Following molecular identification using specific bacterial

gene primers, the group of brains from the polymicrobial infections failed to detect *P. gingivalis* genomic DNA at 12 weeks. However, by 24 weeks 2 out of 12 ApoE$^{-/-}$ mice brain specimens demonstrated the presence of *P. gingivalis* genomic DNA (Fig. 1 Panel f). The brain tissue sections from polymicrobial-infected mice did not show the presence of *T. denticola* and *T. forsythia* at either 12 weeks or 24 weeks (Table 3).

Immunoblot analysis of infected mouse brain tissue

~~Immunoblotting was performed according to Poole *et al.*, [15] on all brain specimens with the anti-*P. gingivalis* (Clone 1B5 which detects both LPS and gingipains) antibody, the s-layer (*T. forsythia*) antibody and the anti-*T. denticola* ATCC 35405 antibody against FhbB protein. Positive controls demonstrated appropriate bands at the expected molecular weights. The anti-*P. gingivalis* (Clone 1B5) antibody demonstrated a ladder of bands in the range of 45-12 kDa as reported by Poole *et al.*, [15]. A single band at 11.4 kDa was detected for anti-*T. denticola* antibody against FhbB protein [47], and anti-*T. forsythia* antibody against the s-layer demonstrated multiple bands with two prominent bands at 230 and 270 kDa [48]. However, n~~None of the test tissue lysates demonstrated LPS, FhbB protein, and the S-layer protein from their respective bacterial species in the mono- and polymicrobial-infected groups (data not shown).

Histology of the infected mouse brain

Overall morphological observations of the temporal lobe, including the hippocampus, appeared well preserved in H/E preparations obtained from all brains (Fig. 2). The pyramidal neurons in all sub-regions of the hippocampus (CA1-CA4) and the dentate gyrus in sham-infected and infected brains generally also appeared to be well preserved (Fig. 2 a-d). Occasionally, shrunken and darker neurons were noted to a varying extent in CA1-CA4 regions and the dentate hilus with a random distribution (not shown). There were no abscesses in the brain and there were no signs of the classical blood-borne inflammatory

cells (neutrophils, lymphocytes) or sites of focal hemorrhage. Thioflavin T and methenamine silver, neutral staining methods failed to demonstrate any evidence for the presence of either Aβ4 plaques or NFTs in the hippocampus or in the fronto-temporal cortex regions in all of the brains examined.

Immunofluorescence detection of periodontal pathogens in infected mouse brain tissue

Cell markers associated with glial cell activation

Astrocytes (GFAP)

All the sections from the sham-infected brains and mono- and polymicrobial--infected groups in which the primary antibody was omitted remained negative (Fig. 3a and d). Immunolabeling of sections for GFAP in the sham-infected control brains demonstrated numerous astrocytes with activated phenotypes around the lateral ventricles (Fig. 3b) as well as scattered astrocytes within the hippocampus CA1-CA4 regions at both time points (Fig. 3c). The brain tissue sections from *P. gingivalis* mono-bacterial--infected groups at 12 and 24 weeks showed astrocytes at the periphery of the lateral ventricles (Fig. 3e) and within the hippocampus (Fig. 3f). There was no statistical difference when cells/area were counted and compared with the sham-infected mice. The brain tissue sections from *T. denticola* mono-infected groups at 12 and 24 weeks demonstrated a similar density of astrocytes scattered at the periphery of the lateral ventricles and within the hippocampus (not shown) as observed in the *P. gingivalis*--infected and sham-infected mice. The brain tissue sections from *T. forsythia* mono-infected groups at 12 and 24 weeks demonstrated a lower density of astrocytes scattered at the periphery of the lateral ventricles and within the hippocampus compared with the *P. gingivalis* and *T. denticola* groups as well as the sham-infected mice (not shown). Equally, the polymicrobial--infections demonstrated no significant difference compared with the control group. GFAP labeling was observed in the circumventricular regions as well as in the hippocampus (not shown).

Microglia (Iba 1)

All mouse brain sections in which the primary antibody was omitted remained negative for microglial cell distribution (Fig. 4a and d). Only a few microglial cells were observed following immunolabeling of sections with the Iba 1 antibody around the lateral ventricles at 12 and 24 weeks in the sham-infected brain sections (Fig. 4b), with even fewer cells (mainly processes, Fig. 4c) in the hippocampus. Similar microglial cell distribution was observed in the *P. gingivalis*–infected brains around the lateral ventricles (Fig. 4e), and few microglial cell bodies with branched processes were observed in the hippocampus (Fig. 4f). The brain tissue sections from *T. denticola* mono-infected groups at 12 and 24 weeks demonstrated no differences in the density of microglia scattered around the periphery of the lateral ventricles or within the hippocampus (not shown). Similarly, there were no differences observed between sham-infected, *T. forsythia*–infected, and polymicrobial–infected brain sections.

Detection of bacterial virulence factors in infected mouse brain tissue

Immunolabeling of brain cryo-sections was unable to demonstrate the presence of any of the three bacteria used for infection when tested using anti-*P. gingivalis* antibody, rabbit antisera against *T. forsythia*, and anti-*T. denticola*.

Detection of complement activation proteins in mouse brain tissue

The sham-infected mouse brain sections, in which the primary antibody was omitted, remained negative for C3 complement activation products (Fig. 5a and 6a). Intracellular labeling detected complement activation products for the common C3 component activation fragments (iC3b, C3b and C3d) (Figs. 5b, 6b) and the membrane attack complex C9 neoepitope (Fig. 6c), specifically on microglia rather than on astrocytes and/or neurons from all brain tissues in sham-infected mice. The complement activation products for the common C3 components (iC3b, C3b and C3d) and C9 (C9 neoepitope) were detected in *P. gingivalis*-infected mouse brains (12 weeks), but the labeling was intracellular and exclusive to

microglia. By 24 weeks, the glial cell labeling was still high (Fig. 5c), but C3 (Fig. 6d and e) and C9 (Fig. 6f) activation fragments appeared to be opsonized onto pyramidal neurons, particularly in the CA2 area of the hippocampus in 4 out of 12 infected brains (P = 0.032). Labeling of the C9 neoepitope was observed in 2 out of 12 specimens (P > 0.05, Fig. 6f). In contrast, both *T. denticola* and *T. forsythia* infections (12 weeks) were similar to the control mice, demonstrating intracellular staining in microglial cells. However, at 24 weeks, 1 out of 12 from each group demonstrated both C3 (iC3b, C3b and C3d) and C9 neoepitope localized to CA neurons (P > 0.05) (data not shown). Immunolabelling of polymicrobial-infected mouse brains (12 and 24 weeks) with the same antibodies also demonstrated the glial cells.

Discussion

Infectious agents have previously been linked to cognitive decline [9-13], and more recently periodontal pathogens and/or their virulence factors have been implicated in the development of AD [14, 15]. This study explored the hypothesis that infectious agents and/or their components from oral diseases such as periodontitis can access the brain and contribute to local CNS inflammation that eventually leads to the development of a chronic inflammatory component of AD. In this study we investigated the possibility that oral pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* can access the brains of ApoE$^{-/-}$ mice following experimental induction of periodontitis as mono- as well as polymicrobial ~~-~~infections. *F. nucleatum* has the ability to co-aggregate with early colonizers in the oral cavity as well as the late colonizers such as *P. gingivalis*, *T. denticola*, and *T. forsythia* [36-38]. ~~In addition, abscesses in the human brain have been reported to be caused by *F. nucleatum* [39].~~ However, in the present study no attempt was made to detect *F. nucleatum* in the brain specimens as *F. nucleatum* is part of another ongoing study. The significance of using a periodontal disease model to assess AD lies in understanding the role of bacteria accessing

the brain and thereby priming glial cells to mount a subsequent local immune response and contribute to neuronal lysis. One previous study, which was performed with an endodontic infection model using wild-type and the severe-combined-immunodeficiency (SCID) mice, demonstrated that only the SCID mice were conducive to *T. denticola* invasion following mono- and polymicrobial-infections [36]. That study showed that *T. denticola* can disseminate to distant body organs, including the brain, heart, and spleen while *P. gingivalis* and *T. forsythia* were undetected [3640]. In our current study using a periodontal infection model in ApoE$^{-/-}$ mice, we report a contrasting finding in which we observed the dominance of *P. gingivalis* in accessing the brain in comparison to *T. denticola* and *T. forsythia*. These differences in our study from those of Foschi et al., [3640] maybe due to the bacterial strains used, the dosage of infection administered, method of inoculating animals during infection, differences in disease models (endodontic vs periodontal disease), as well as the genetic makeup of the mice used. For example, the only common strain between this study and that of Foschi et al., [3640] is *T. forsythia* (ATCC 43037) and the dose of bacteria used in each study was different (higher by a factor of 10 in this study). Based on the available data it is likely that *T. forsythia*, being a non-motile bacterium which lacks fimbriae, is unable to transmigrate to the brain [48]. We found that *P. gingivalis* FDC381 DNA predominated in the brains of ApoE$^{-/-}$ mice, and this strain is highly fimbriated compared to the *P. gingivalis* ATCC 33277 [489], used by Foschi et al., [3640]. Although, both strains of *T. denticola* (ATCC 35404 and ATCC 35405) are motile, the *T. denticola* (ATCC 35405) used by Foschi et al., [3640] at a lower dose disseminated to the brain. This difference may be attributed to the outer membrane, with abundant pore-forming adhesion protein that may be lacking in our *T. denticola* (ATCC 35404) strain [4950]. Thus, the virulence of the bacteria may have contributed to its accessibility to the brain, rather than being a dose--dependent effect. especially in the mono-bacterial infected group of mice. However, a dose dependent effect

~~may be exaggerated in the polymicrobial infection model as the absolute numbers of all bacteria in the inoculum were different to that used in mono-bacterial infections (10^9). Human periodontal pathogens are not habitants of the murine oral cavity therefore the antibiotic pretreatment was used once only and prior to the first infection to facilitate initial colonization of the human periodontal pathogens to the teeth and gingival surfaces of the experimental mice.~~

Despite the differences in bacterial strains used and their dosage, as well as the genetics of the experimental animals, our results show that *P. gingivalis* strain FDC 381 used to infect the oral cavity of the ApoE$^{-/-}$ mice was able to access the brain tissue, providing definitive evidence for transmigration of this bacterial species from the oral cavity to the brain. The fact that more brains demonstrated a greater *P. gingivalis* infection at 24 weeks of infection suggests that the translocation of bacteria is likely to be time dependent. Inflammation occurring at 24 weeks of infection may be increasing the permeability of the blood-brain barrier and facilitating easier access of bacteria into the brain.

~~The circumventricular organs are not part of the diagnostic criteria for AD [51]. However, we analyzed these tissues for bacterial identification to keep in with our human brain study, in which the only tissue available to us was from the lateral ventricle of the parietal lobe [15]. In addition, the hippocampus from the frozen tissue was reserved for fixation sensitive antibodies such as those used for detecting complement activation. Finding molecular evidence of~~ Detecting *P. gingivalis* in the Apo E$^{-/-}$ mice brains in this *in vivo* study supports the data presented in our recently published study of human brain specimens in which we detected *P. gingivalis*~~-~~specific LPS in 4 out of 10 AD human brains [15]. Together these studies provide evidence to support an association between periodontal disease and AD. When examined for general morphological preservation of the fronto-temporal lobe, including the hippocampus, rehydrated paraffin wax sections showed no signs of abscess

formation, ~~The fact that there were no signs of any abscess formation in the brain suggests that *P. gingivalis*, if metabolically active in the brain, may take several years to form an abscess as is the case with non-oral bacteria such as *Propionibacterium acnes* which can take 10 years to form abscess following entry into the brain [52]. In addition, there were~~ no myeloid lineage cells (neutrophils, lymphocytes) infiltrating into the brain, and no sites of focal brain hemorrhage.

~~Bacterial virulence factors were not detected in any of the brains by immunoblotting and/or by immunolabeling with the aforementioned antibodies. Although this appeared surprising at first, however, the lack of detection may be attributed to the inability of these bacteria to access the brain~~

Our immunoblotting and immunofluorescence techniques with specific antibodies did not show the presence of bacterial virulence factors in any of the brain tissues examined. If any of these are metabolically active in the brain, it may take several years to form an abscess as seen in the case with non-oral bacteria such as *Propionibacterium acnes*, which can take 10 years to form an abscess following entry into the brain [50]. Although this appeared surprising at first, the lack of detection may be attributed to the inability of these bacteria to access the brain due to their rapid clearance from the systemic circulation and/or they were neutralized upon entry by the already enhanced microglial cell inflammatory phenotype in these mice [51~~3~~, 52~~4~~]. Another possible reason may be that the antibodies themselves failed to detect their epitope in tissue sections or the antigen itself was below the detection limit of both immunoblotting and immunolabelling.

We focused on the hippocampus region of the brain to detect any early cellular changes in the Apo-$E^{-/-}$ mice brains, as according to Braak and Braak [53~~1~~]~~,~~ neurodegeneration begins in the entorhinal cortex and spreads to the hippocampus followed by other regions. Screening for the AD hallmark associated structures by thioflavin T and

methenamine silver methods failed to provide any evidence for the fibrillar Aβ4 and NFTs in the entorhinal cortex or the hippocampus regions. A plausible reason for the inability to detect the AD hallmark proteins could be the relatively short time span of chronic infection in our mouse model because, even in the accelerated transgenic AD animal model and in the AβPP and SS-1 transgenic mice, insoluble Aβ4 deposition and plaque formation usually takes between 6 to 12 months [545, 556]. Further, ApoE$^{-/-}$ mice used in the current study are unlikely to demonstrate Aβ4 deposition as they lack the essential protein required for amyloid to form insoluble fibrils [3740]. Hence it will be beneficial for a future study to be designed with a longer duration of mono- and polymicrobial--infection in a non-ApoE$^{-/-}$ rodent model so as to demonstrate the direct link between periodontal disease and AD hallmark proteins.

Previous studies with ApoE$^{-/-}$ mice have identified glial cell activation in which microglia demonstrate evidence of an increased secretion of cytokines, especially of tumor necrosis factor –alpha (TNF-α) [513, 524], a cytokine of macrophage origin. This observation has been suggested as an impaired immuno-modulatory function of macrophages in controlling the innate immune responses in this animal model [567-589]. Microglial cells are the tissue--bound macrophages of the brain capable of expressing a range of proinflammatory cytokines and phagocytosing cellular debris to reduce the inflammatory response to pathogens. However, the finding that the ApoE$^{-/-}$ mice have higher levels of endogenous proinflammatory cytokines, especially TNF-α, suggests that it is likely that microglia were already in their primed phenotype. In this study we also found responsive fibrillary astrocytes, particularly at the peri-circumventricular organ sites following initial microglial cell activation. Complement is a pivotal pathway in the CNS innate immune response following infections. It is part of the body's innate immune defense mechanism and has been reviewed elsewhere [60-62]. In the CNS, the dominant mode of complement activation is the classical pathway where neurons show vulnerability to complement mediated

damage [5963] and microglia synthesize complement proteins [604]. Hence, we set out to detect any evidence for the activation of the common C3 and the terminal pathway of complement leading to the formation of the membrane attack complex in our infected mice brain specimens. Our study demonstrated an intracellular localization of C3 and C9 exclusively in microglia in all brains, suggesting that these cells were actively synthesizing complement components [604] rather than being opsonized with the complement activation fragments, again supporting the view that microglia were already in their primed/activated state [513, 524, 615].

However, our observation of the cell surface membrane staining of C3 activation fragments (iC3b, C3b and C3d) and the membrane attack complex (anti-C9 neoepitope) exclusively on CA pyramidal neurons of the mono- and polymicrobial--infected mice at 24 weeks but not at 12 weeks suggests that the inflammatory burden was increasing from protection to causing bystander injury on complement activated neurons. ~~The C3 activation fragments opsonized to neurons in the *P. gingivalis* mono-infected group were statistically significant whereas the observed membrane attack complex detected on neurons in the same group did not reach significance.~~ In view of us detecting C3 activation fragments being opsonized on the pyramidal neurons, it appears likely that bacteria (*P. gingivalis*) and/or its DNA may have triggered the complement activation in these infected mice.

Our study supports the observation from previous studies which hypothesized that bacterial infections would contribute to the development of AD pathology via mechanisms involving acute--phase proteins, including cytokines and the complement cascade in which neurons would be attacked [31-33]. The presence of cytokines and activated complement cascade can be used as a marker to represent local CNS inflammation [1, 33].Thus, the demonstration of activated complement cascade here in response to *P. gingivalis* directly

infecting the brain supports the conclusion that chronic local inflammation constitutes a component of developing AD pathology.

~~An investigation from our collaborators conducted on the same set of animals used in this study demonstrated that *T. denticola* mono-bacterial infection-induced significant atherosclerosis risk factors (cholesterol, very low-density lipoprotein or VLDL and, serum oxidized LDL), and acute phase protein serum amyloid A, as well as a significant decrease in endothelial dysfunction marker (nitric oxide) [42]. At the same time, this study using the brains from the same mice provides some evidence towards confirming this hypothesis, alongside novel data demonstrating the transmigration of *P. gingivalis* from the oral cavity to the brain of ApoE$^{-/-}$ mice where they can initiate local innate immune responses including complement activation and neuronal damage.~~ Finally, this study demonstrates that, in the absence of fibrillary Aβ4 deposition the neurons remain vulnerable to complement mediated damage from *P. gingivalis* accessing the brain.

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TABLE 1a: PCR primers from Paster et al.~~,~~ [43]

Primer	Function	Orientation	Sequence
D88	PCR	Forward	GAGAGTTTGATYMTGGCTCAG
E94	PCR	Reverse	GAAGGAGGTGWTCCARCCGCA

TABLE 1b: Specific primer sets used for analysis of bacterial DNA from ApoE$^{-/-}$ mice brains by PCR

Primer & Reference	Amplicon size	Primer	Sequence
P. gingivalis [44]	PCR	Forward	AGGCAGCTTGCCATACTGCG
P. gingivalis [44]	PCR	Reverse	ACTGTTAGCAACTACCGATGT
T. denticola [415]	PCR	Forward	TAATACCGAATGTGCTCATTTACAT
T. denticola [415]	PCR	Reverse	CTGCCATATCTCTATGTCATTGCTCTT
T. forsythia [44]	PCR	Forward	GCGTATGTAACCTGCCCGCA
T. forsythia [44]	PCR	Reverse	TGCTTCAGTGTCAGTTATACCT
M13 (Invitrogen)	Sequencing	Reverse	CAGGAAACAGCTATGAC

TABLE 2: Source of antibodies and their working concentration and/or dilutions used

Antibody	**Supplier**	**Final conc/ dilution**
Rabbit anti-GFAP (gift)	Dr Jia Newcombe (The Multiple Sclerosis Society Laboratory, UK)	1/1000
Goat anti-Iba 1 (ab5076)	Abcam	1/250
Mouse anti-*P. gingivalis* (Clones 1B5) tissue culture supernatant (gift)	Prof. Michael A. Curtis (London, UK)	1B5 1/10,
Rabbit anti-*T. forsythia* (S-layer protein)	Dr Graham Stafford (University of Sheffield, UK).	1/20,000
Rat anti-*T. denticola* (FhbB protein)	Prof. Thomas T. Marconi, (USA)	1/5000
Blocking solution	0.01 M phosphate buffered saline (PBS) pH 7.3 containing 0.01% normal goat or rabbit serum and 0.25% tween 20	-
Normal serum: goat (X0907), rabbit (X0902).	DakoCytomation, Germany,	0.01%
Rat anti-mouse C3b/iC3b/C3d	Hycult Biotechnology, UK	1/50
Rabbit anti-rat C9 neoepitope	Professor B. Paul Morgan, and Dr Timothy R. Hughes, Cardiff University.	1/100

TABLE 3: DNA detected from periodontal pathogens in the ApoE$^{-/-}$ mice brains

Mono infections	**DNA detected at 12 weeks**	**DNA detected at 24 weeks**	**Polymicrobial infections 12 weeks**	**Polymicrobial infections 24 weeks**
Sham--infected	0 out of 12	0 out of 11	0 out of 11	0 out of 11
P. gingivalis	6 out of 12, P = 0.006	9 out of 11 P = 0.0001	0 out of 11	2 out of 11
T. denticola	0 out of 12	0 out of 12	0 out of 11	0 out of 11
T. forsythia	0 out of 12	0 out of 12	0 out of 11	0 out of 11

Legends

Poole *et al.*, Figure 1

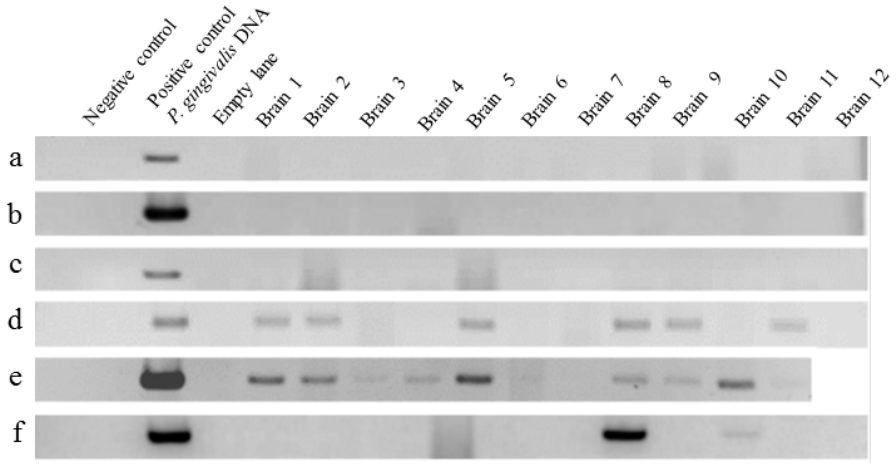

Figure 1: Molecular identification of *P.gingivalis* in brain tissue sections using specific primers. Panels a and b) mono sham-infected group 12 and 24 weeks, c) polymicrobial sham-infected group 24 weeks, d) Mono- infection with *P. gingivalis* at 12 weeks, e) Mono-infection with *P. gingivalis* at 24 weeks, f) Polymicrobial infection with *P. gingivalis* at 24 weeks. d) Lanes corresponding to Brain 1, 2, 5, 8, 9, 11 demonstrated a band at 400bp. P value = 0.006. e) Lanes corresponding to Brain 1, 2, 3, 4, 5, 6, 8, 9, 10, 11 demonstrated a band at 400bp. P value = 0.0001. f) Lanes corresponding to Brain 8 and 10 demonstrated a band at 400bp.

Poole *et al.*, Figure 2

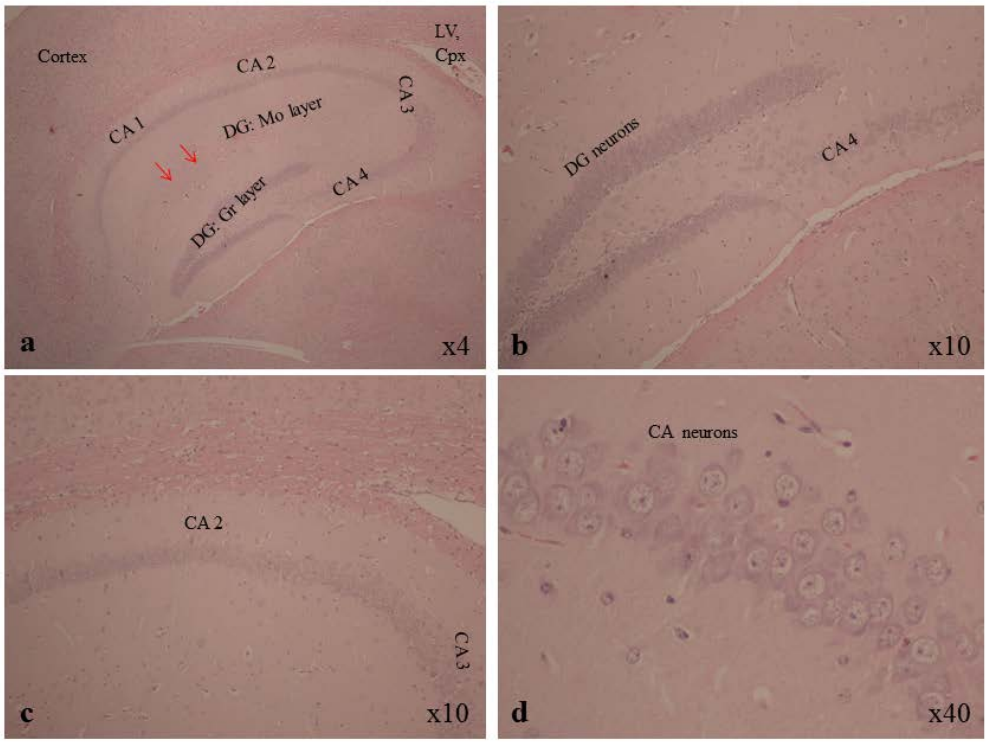

Figure 2: Haematoxylin and Eosin stained tissue section from the temporal lobe of Apo-E$^{-/-}$ mice demonstrating the overall preservation of a) CA1-CA4 regions of the hippocampus, b) Higher magnification of the dentate gyrus neurons, c) the cortical and hippocampal fissure by the lateral ventricle in relation to CA2 and 3 neurons, d) higher magnification of the CA2 neurons. DG: Gr layer = dentate gyrus granule cell layer. The red arrows depict fused hippocampal fissure. LV = lateral ventricle containing the choroid plexus.

Poole *et al.*, Figure 3

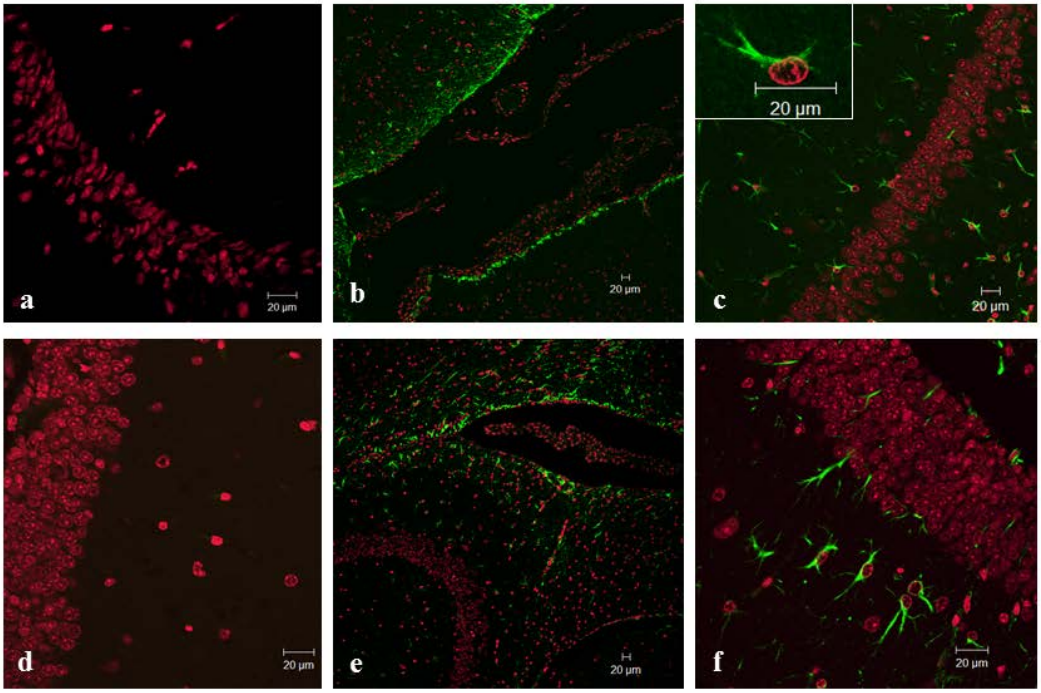

Figure 3: Immunolabelling of the temporal lobe of ApoE$^{-/-}$ mice with rabbit anti-human GFAP to assess astrogliosis. a and d) negative control images whereby primary antibody is omitted. Sham--infected (b, c) in which (b) demonstrated abundance of immunopositivity especially around the periphery of the lateral ventricles and the inset in (c) shows the morphology of cells labeled with anti-GFAP. These appeared as fibrillary astrocytes with reactive phenotype. The mono *P. gingivalis* infected (e, f) brains at 24 weeks demonstrated a more widespread distribution of fibrillary astrocytes around ventricles but their distribution within the hippocampus region was similar to that observed in the sham--infected brains.

Poole *et al.*, Figure 4

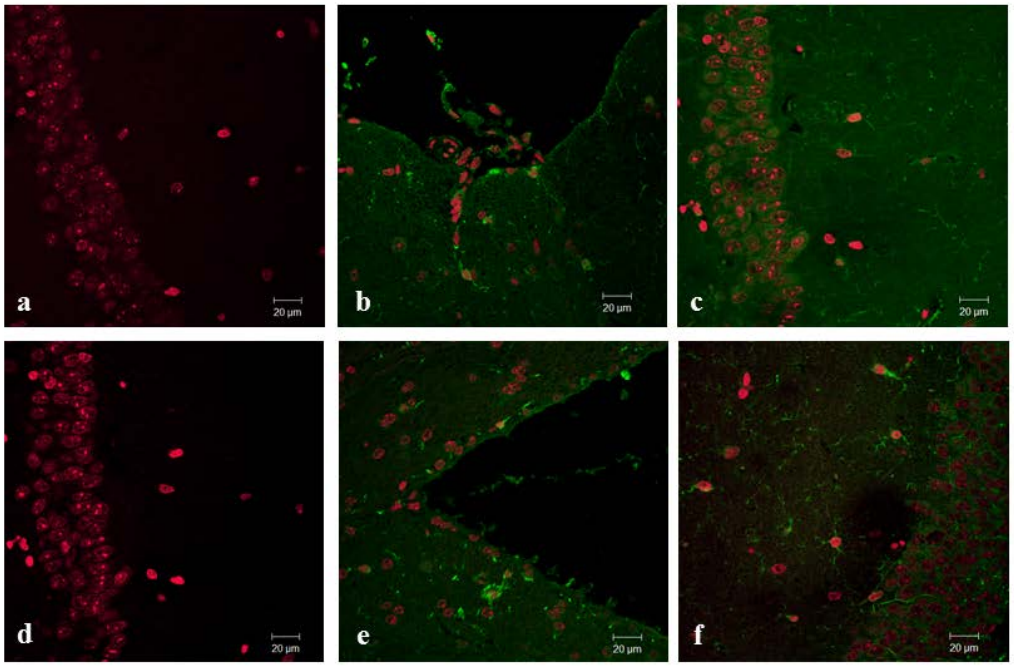

Figure 4: Immunolabelling of the temporal lobe of ApoE$^{-/-}$ mice with goat anti-mouse Iba1 antibody to assess microgliosis. a and d) negative control images whereby primary antibody is omitted. Sham--infected (b, c) in which (b) demonstrated immunopositivity around the periphery of the lateral ventricles. The mono- *P. gingivalis* 24 weeks infected (e, f) brains demonstrated similar labeling to that observed in the sham--infected brains, in both the lateral ventricles and hippocampal regions.

Poole *et al.*, Figure 5

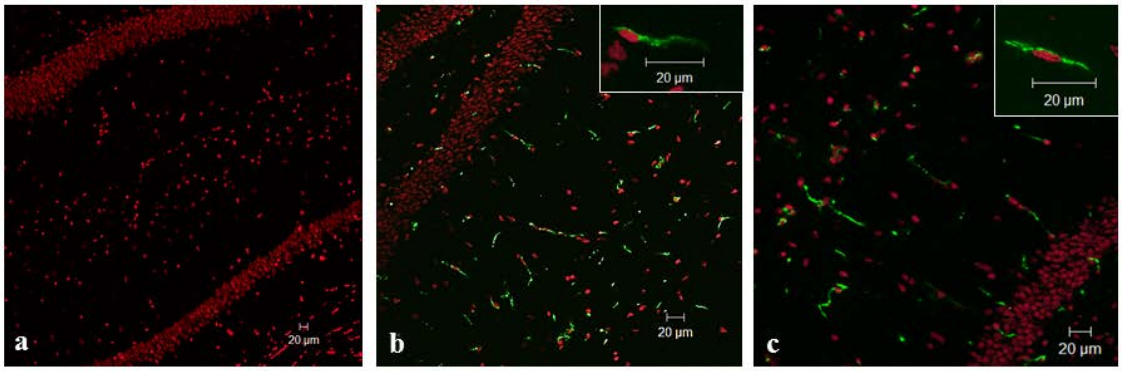

Figure 5: Cryo-section from the temporal lobe of ApoE$^{-/-}$ mice immunolabelled for complement activation fragments in the hippocampus using rat anti-mouse C3b/iC3b/C3d. (a) Control, where the primary antibody was omitted from the tissue section. In both sham-infected (b) and infected (c) brains, the labeling appears intracellular within branched microglia demonstrating an activated phenotype. The inset (b-c) shows the branched morphology of cells labeled with the same antibody.

Poole *et al.*, Figure 6

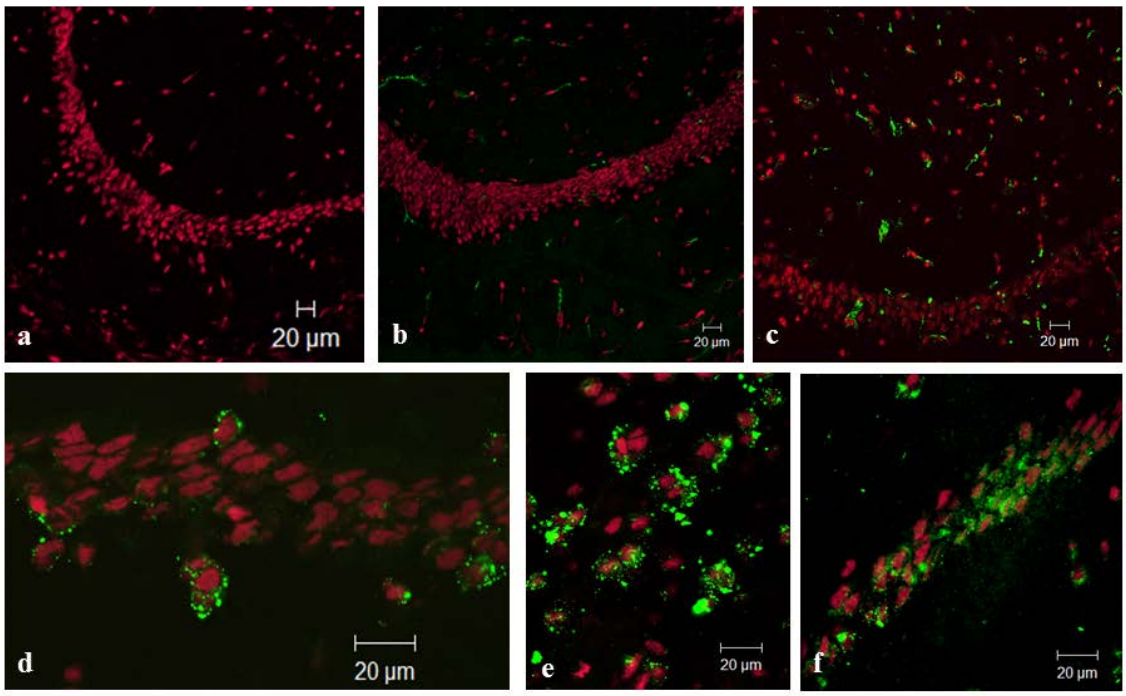

Figure 6: Immunodetection of complement fragments in brain tissue sections using rat anti-mouse C3b/iC3b/C3d. (a) Negative control (b-c) sham--infected brains with rat anti-mouse C3b/iC3b/C3d (b) and rabbit anti-rat C9 neoepitope (c). (d-f) *P. gingivalis* infected brain with rat anti-mouse C3b/iC3b/C3d (d and e) and rabbit anti-rat C9 neoepitope (f); showing labeling on the cell surface membranes of the CA neurons in the infected brains (P = 0.032).