Review paper

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Lipopolysaccharide-stimulated, NF-kB-, miRNA-146a- and miRNA-155-mediated molecular-genetic communication between the human gastrointestinal tract microbiome and the brain

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Abstract

Through the use of RNA sequencing, microRNA (miRNA) and messenger RNA (mRNA) microfluidic array analysis, LED Northern, Western and ELISA analysis and multiple bioinformatics algorithms we have discovered a novel route for pathogenic communication between the human gastrointestinal (GI)-tract microbiome and the brain. The evidence suggests that this pathogenic gut-brain circuit involves: (i) lipopolysaccharide (LPS) from the GI-tract resident enterotoxigenic Gram-negative bacteria Bacteroides fragilis (BF-LPS); (ii) LPS transit across the GI-tract barrier into the systemic circulation; (iii) transport of a highly pro-inflammatory systemic BF-LPS across the blood-brain barrier (BBB) into the brain-parenchyma and neuronal-cytoplasm; (iv) activation and signaling via the pro-inflammatory NF-kB (p50/p65) transcription-factor complex; (v) NF-kB-coupling and significant up-regulation of the inducible pro-inflammatory microRNA-146a (miRNA-146a) and microRNA-155 (miRNA-155); each containing multiple NF-kB DNA-binding and activation sites in their immediate promoters; and (vi) subsequent down-regulation of miRNA-146a-miRNA-155 regulated mRNA targets such as that encoding complement factor H (CFH), a soluble complement control glycoprotein and key repressor of the innate-immune response. Down-regulated CFH expression activates the complement-system, the major non-cellular component of the innate-immune system while propagating neuro-inflammation. Other GI-tract microbes and their highly complex pro-inflammatory exudates may contribute to this pathogenic GI-tract-brain pathway. We speculate that it may be significant that the first Gram-negative anaerobic bacterial species intensively studied as a potential contributor to the onset of Alzheimer's disease (AD), that being the bacillus Bacteroides fragilis appears to utilize damaged or leaky physiological barriers and an activated NF-kB (p50-p65) – pro-inflammatory miRNA-146a-miRNA-155 signaling circuit to convey microbiome-derived pathogenic signals into the brain.

Key words: *Alzheimer's disease, Bacteroides fragilis (B. fragilis), complement factor H (CFH), dysbiosis, microRNA-146a, microRNA-155, NF-kB (p50/p65).*

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Overview: human GI-tract microbiome and *Bacteroides fragilis*

The gastrointestinal (GI) tract of *Homo sapiens* contains a complex microbiome consisting primarily of bacteria, with archaea, fungi, microbial eukaryotes, protozoa, viruses, and other microorganisms making up the balance [3-5,7,8,16,19,25,26,89,90]. Together with human host cells this microbiome comprises the entire meta-organism whose host interactions and symbiotic associations are critical to human health and disease [4,16,18,35,72,88-90]. These diseases include lethal, progressive, age-related, inflammatory neurodegenerative disorders of the human central nervous system (CNS) such as Alzheimer's disease (AD) [4,5,9,35,58,60,72,76,82]. Of the 52 currently recognized bacterial divisions, humans have co-evolved with just 2 dominant phyla: *Bacteroidetes,* representing ~20-30% of all human GI-tract resident bacteria, and *Firmicutes* (about 70-80%), with *Actinobacteria* (~3%), *Proteobacteria* (~1%) and *Verrucomicrobia* (~0.1%) making up the remainder [4,5,7,61,78]. These four major bacterial phyla represent the '*bacterial-core*' of the human GI-tract microbiome [4,7,8,78,82]. The vast majority of all GI-tract microbiota consists of Gram-negative anaerobic bacteria, and *Bacteroidetes* species represent the most abundant Gram-negative anaerobes, outnumbering *Escherichia coli* in abundance by about 100 to 1 [3-8,14,15,61-64]. Certain strains of *Bacteroidetes* species such as *Bacteroides fragilis* (*B. fragilis*), as a normal commensal microbe of the human GI-tract, are thought to be ordinarily beneficial to human health due to their multiple capabilities: (i) to biosynthesize useful metabolic co-factors and products such as polysaccharides, transport proteins, volatile fatty acids and other nutrients [9,14,47,62,74]; (ii) to cleave dietary fiber into digestible short-chain fatty acids (SCFAs) that include acetate, propionate, and butyrate [9,38,63,74]; (iii) to function in the maintenance, development and homeostasis of the host immune system [14,47,62,74,79]; (iv) to support immunomodulation and protection against pathogens including potentially pathogenic GI tract bacteria [9,14,29,63,79]; and (v) to support glucose homeostasis [8,9,13,63,69,72]. Conversely, when enterotoxigenic strains of *B. fragilis* or their array of secretory neurotoxins leak through normally protective biophysiological-mucosal barriers they can cause substantial inflammatory pathology systemically that can contribute to significant mortality and morbidity [15,29,57,63,72,90]. Dietary intake of fiber may have a determinant role in regulating the composition, organization and stoichiometry of the GI-tract microbiome; for example *Bacteroidetes* species proliferate in porcine models fed high-fat diets that are deprived of sufficient dietary fiber [13,22,32,57,69,83,84].

The secreted LPS derived from the outer membrane of *B. fragilis* (BF-LPS) is a remarkably neurotoxic and pro-inflammatory lipid-sugar lipoglycan consisting of a hydrophobic domain known as a lipid A '*endotoxin*', a nonrepeating "core" oligosaccharide, and a distal polysaccharide '*O-antigen*' [12,77]. Interestingly, of several different AD-associated pro-inflammatory mediators – including the pro-inflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), the neurotoxic Aβ40 and Aβ42 peptides, the combination of Aβ42 + IL-1β together, the LPS isolated from *E. coli* (EC-LPS) or *B. fragilis* (BF-LPS) – that were tested for their ability to induce the pro-inflammatory transcription factor NF-kB (p50/p65 complex) in human neuronal-glial (HNG) cells in primary co-culture, BF-LPS was by far the most potent. For example, 25 nM doses of BF-LPS administered to HNG cells elicited an ~11-fold more of a pro-inflammatory response than the same quantity of Aβ42 peptide alone and a remarkable ~27-fold more of an inflammatory response than the pro-inflammatory cytokine TNF-α alone [12,46,47]. In addition to lipooligosaccharide (LOS) and lipopolysaccharide (LPS) generation, *B. fragilis* endotoxins are a leading cause of anaerobic bacteremia and sepsis driving systemic inflammatory distress through their generation of the highly pro-inflammatory ~20 kDa heat-labile zinc-dependent metalloprotease *B. fragilis* toxin (BFT) *fragilysin* [10,14]. Exposure to BFT results in the infiltration of a variety of inflammatory cells and also in the destruction of the mucosal epithelial cell layer [10,62,63]. The ability of *B. fragilis* strains to secrete BFT classifies these microbes as: (i) those that do not secrete BFT as the nontoxigenic form of *B. fragilis* (NTBF); and (ii) those that do secrete BFT are classified as the enterotoxigenic form of *B. fragilis* (ETBF) [14,62]. Interestingly, the ETBF form can (i) disrupt epithelial cells of GI tract barriers via cleavage of the synaptic adhesion zonula adherens protein E-cadherin [14,24,39,62,63,65]; and (ii) induce clinical pathology, including intestinal diarrhea, celiac disease and systemic inflammation

[10,25-28,46,47,54,62,65,80]. Indeed, under pathophysiological conditions within the aging AD brain it might be expected that a pro-inflammatory cocktail of BF-LPS, BFT and other *B. fragilis*-derived exotoxins, amyloids and small non-coding RNAs (sncRNAs) would assault normal brain homeostasis together to elicit an even greater pro-inflammatory response in driving the neurodegenerative disease process (Fig. 1). It should also be kept in mind that other GI-tract microbiome-resident microbes including fungus, protozoa, viruses, and other commensal microorganisms may also contribute highly neurotoxic exudates that can be strongly detrimental to the homeostasis of aging CNS neurons.

Systemic inflammation and the propagation of GI-tract microbiome-derived signals

GI-tract microbiome-derived neurotoxin entry into the systemic circulation may be a precursor event to the onset of inflammatory neurodegeneration in the CNS and establishing pathogenic pathways of communication between the systemic and central innate-immune systems [23,29,31,66]. It is currently not well understood if GI-tract barrier-disrupting proteolytic endotoxins such as BFT are able to propagate pathogenic actions via the systemic circulation to further disrupt the blood-brain barrier and transfer LPS, BFT, and other endotoxins into the cerebrovascular circulation to the brain parenchyma, neural cells and synaptic circuitry of the CNS. However, it has recently been reported that BF-LPS, BFT and amyloid peptides progressively alter neural cytoarchitecture, synaptic adhesion, affecting both the function and integrity of synapses – a series of processes that play critical roles in the disruption of functional inter-neuronal signaling throughout neuronal networks in AD [33,39,42,48,57,65,76].

Up-regulated microRNAs (miRNAs) and down-regulation of essential signaling components in Alzheimer's disease by GI-tract microbiome-derived LPS

The structure, function and evolution of miRNAs including their expanding list of critical regulatory roles in CNS development and age-related human neurodegenerative diseases such as AD have been extensively reviewed and will not be dealt with further here [1,2,34,41,45,49,51,70,83,88]. Because miRNAs are relatively unstable in brain and retinal tissues with half-lives on the order of ~1-3 hrs, only significantly up-regulated miRNAs have been studied in short post-mortem interval (PMI) tissues of PMIs of 2-3 hrs or less; down-regulated miRNAs may simply be a consequence of the highly oxidative and pro-inflammatory degradative environment of actively degenerating neural tissues [11,59,67]. It has become abundantly clear that the major mode of action of miRNAs is to function as negative regulators of messenger RNA (mRNA)-mediated gene expression by binding to complementary ribonucleotide sequences in the 3'-untranslated region (3'-UTR) of target mRNAs, thus causing the repression of translation and/or degradation of that target mRNA [21,59,73]. Briefly, a small family of NF-kB (p50/p65 complex) induced pro-inflammatory microRNAs (miRNAs), including miRNA-9, miRNA-34a, miRNA-125b, miRNA-146a, and miRNA-155, ultimately bind with the 3'-UTR of several target messenger RNAs (mRNAs) and thereby decrease their expression. Down-regulated mRNAs include those encoding the SH3-proline-rich multi-domain-scaffolding protein of the postsynaptic density (SHANK3), the triggering receptor expressed in myeloid/microglial cells (TREM2) and complement factor-H (CFH); similar deficiencies in these 3 proteins are also observed in sporadic AD brain [20,27,28,36,89,90]. The critical importance of a down-regulated CFH mRNA and protein, and hence the observed deficits in CFH expression and abundance in AD in this scheme due to up-regulated pro-inflammatory miRNA-146a and miRNA-155 is particularly significant. CFH down-regulation is further discussed below due to its critical role in the regulation of the innate-immune response and the onset and propagation of neuro-degeneration and inflammatory signaling [16,36,43,50,52,53,55] (Fig. 1).

Complement factor H as an important LPS-NF-kB-miRNA-146a-miRNA-155 end target

Complement factor H (CFH; GC01P196621), encoded from *the regulator of complement activation gene cluster* at human chromosome 1q31.3, is a large, structurally versatile and critical 155 kDa soluble glycoprotein moderately expressed in the human neocortex and retina, and highly expressed in the gall bladder and the liver where the mature plasma form of CFH passes into the systemic circulation [36,50,52,53];

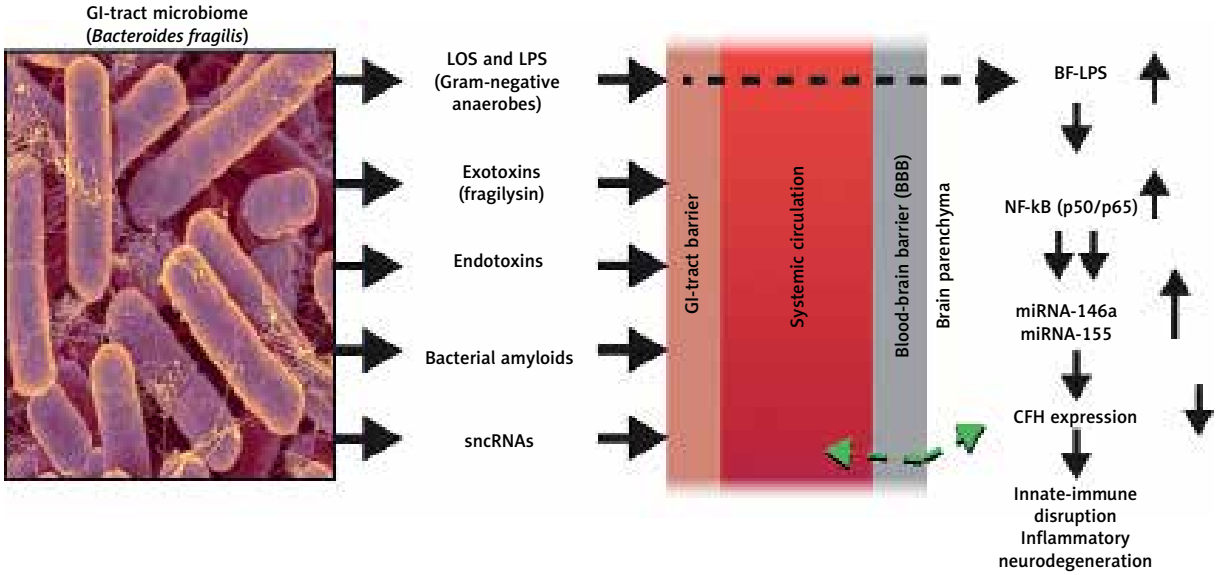

Fig. 1. Gram-negative anaerobes such as the abundant human gastrointestinal (GI)-tract resident *Bacteroides fragilis* (left panel; scanning electron microscopy (SEM) photograph of *B. fragilis* shown (9100×); original micrograph courtesy of Rosa Rubicondior (http://rosarubicondior.blogspot.com/2014/11/evolving-cooperation-but-for-who-or-what.html; last accessed 4 July 2019), when stressed, release a broad spectrum of highly pro-inflammatory and potentially pathogenic molecules into the surrounding medium; these molecules comprise five major classes of secreted neurotoxins that include lipooligosaccharide (LOS) and lipopolysaccharide (LPS), exotoxins (such as fragilysin), endotoxins, bacterial amyloids, and small non-coding RNAs (sncRNAs) [7,12-14,24-27,87]. Normally, a highly dynamic GI-tract barrier keeps bacteria and their neurotoxic exudates compartmentalized within the GI-tract; however, with aging and disease these barriers become 'leaky' and the same neurotoxins can easily transit GI-tract barriers to enter the systemic circulation. Gram-negative bacterial-derived LPS or other bacterial-derived neurotoxins in the systemic circulation give rise to a '*systemic inflammation*' which may be a '*precursor event*' to the development and/or establishment of Alzheimer's disease (AD) and other forms of progressive inflammatory neurodegeneration [16,17,23,26,27,31,37,38,56,57,71,87]. The extremely pro-inflammatory LPS of *B. fragilis* (BF-LPS) is exceptionally neurotoxic toward human CNS neurons in primary culture [40,47]. LPS in the systemic circulation can cross the blood-brain barrier (BBB) to access neural-susceptible brain compartments; there is also recent and compelling evidence that in the 5xFAD transgenic murine model of AD, LPS alone induces the opening of aging physiological barriers including the BBB [6,54]. Multiple laboratories have independently reported perivascular LPS localization in brain arteries and LPS accumulation within the brain parenchyma, within brain cell cytoplasm and surrounding neuronal cell nuclei with specific effects on neuron-specific gene expression [12,13,15,26,27,71,80,87-90]. BF-LPS rapidly and efficiently induces the pro-inflammatory NF-kB (p50/p65) complex in cultured human brain cells followed by increases in the abundance of the inducible pro-inflammatory miRNA-146a and miRNA-155 (both of these miRNAs containing active NF-kB-DNA binding sites in their immediate promoter [25-27,40,43,80,87-90]. One energetically favorable miRNA-146a and/or miRNA-155 mRNA target, both confirmed by bioinformatics and miRNA-mRNA-3'-UTR binding luciferase-reporter assay, is the 3'-untranslated region (3'-UTR) of complement factor H (CFH) mRNA resulting in, respectively, the down-regulation of CFH expression [26,27,48,87]. A down-regulated CFH is associated with the disruption of innate-immune signaling that supports inflammatory neurodegeneration and amyloidogenesis [17,34,36,45,55,75,89-91]. Although CFH is easily detected in the brain parenchyma and neuronal cytoplasm it is also highly abundant in the blood serum of the human systemic circulation, as may be some other pathogenic blood-cell-based biomarkers that may include specific kinase mutations and other polymorphisms [17,45,55,68,91; see also https://www.sigmaaldrich.com/catalog/product/sigma/c5813?lang=en&region=US; ENSG000 00000971-CFH/ tissue; last accessed 4 July 2019]. Like for other biomarkers, CFH communication and/or exchange between the brain parenchyma and systemic circulation (green dashed arrow) is not well understood. Other up-regulated brain-enriched microRNAs and their down-regulated mRNA targets may also be involved in multiple pathogenic signaling mechanisms.

https://www.proteinatlas.org/ENSG00000000971-CFH/tissue; https://www.genecards.org/cgi-bin/carddisp. pl? gene=CFH; last accessed 29 March 2019). Normal human plasma concentrations of CFH range between ~200 and 300 ug/ml, the highest of any plasma complement protein, and as such represents the major human complement regulator in human blood, brain and retina [17,55; unpublished]. CFH is composed of ~20 tandem ~60 amino-acid '*complement control protein*' modules each connected by 3-8 amino acid linkers whose principal function is to regulate the alternative pathway of complement system activation, a central component of the innate-immune system's natural defense against pathogens and microbial infection [27,28,36,52,88-90]. As the major immune regulator of the innate-immune system, CFH recognizes pathogen- and damage-associated molecular patterns (DAMPs) and initiates an immune response in coordination with innate and adaptive immunity [36,52,53,88-90]. When activated, the complement system unleashes powerful cytotoxic and pro-inflammatory mechanisms, and thus its stringent control is critical to allow the restoration of immune-homeostasis and the prevention of damage to host-tissues. Significant levels of CFH expression are maintained in the blood, brain and retinal tissues during development and aging, suggesting that this glycoprotein plays a role in protecting these compartments, cells and tissues from indiscriminate complement activation and innate-immune and/or inflammatory damage [16,45,55,88-90]. Brain CFH, retinal CFH and plasma CFH levels have been found to be significantly decreased in mild cognitive impairment (MCI), in late onset AD (LOAD), in age-related macular degeneration (AMD) patients and in autoimmune disease when compared to age-matched controls [17,27-29,45,55,89,90]. The LPS-induced, NF-kB-stimulated pro-inflammatory microRNAs miRNA-146a and miRNA-155 regulate both brain and retinal CFH expression via very strong overlapping complementary RNA binding sites in the CFH 3' untranslated region (3'-UTR); interestingly the entire 232 ribonucleotide sequence of the human CFH mRNA 3'-UTR appears to contain multiple miRNA recognition features and highly complementary miRNA binding sites that may be alternately used in CFH regulation in the brain, retina and other neural or extra-neural tissues [17,27,28,89,90]. Together miRNA-146a and miRNA-155 recognize an overlapping miRNA regulatory control (MiRC) region in the CFH 3'-UTR (5'-TTTAGTATTAA-3') to which either of these miRNAs may interact [17,45]. Progressive, pathogenic increases in specific miRNA binding to the entire 232 nucleotide CFH 3'-UTR appears to be a major regulator of CFH expression down-regulation, and the inflammatory pathology that characterizes both AD and AMD. Besides miRNA-146a and miRNA-155, the involvement of other up-regulated miRNAs in controlling CFH expression is not known and cannot be excluded at this time [17,45,55]. Epigenetic-based gene therapies, including miRNA-based therapeutic strategies that target miRNA-146a and miRNA-155 using anti-miRNA-146a and/or anti-miRNA-155 strategies, and hence the regulation of CFH expression, may be greatly beneficial and advance the protective effects of CFH at inflammatory sites in CNS disease.

Importantly, CFH has several structurally related proteins also encoded at *the regulator of complement activation gene cluster* at human chromosome 1q31.3 that lack relevant complement regulatory activity – these are known as CFH-related (CFHR) proteins [36,53,55]. The balance between the actions of CFH and the CFHR proteins: (i) determines the degree of complement activation and the innate-immune response; and (ii) regulates the neurophysiological roles of CFH and CFHR in CNS health and disease [36,75]. The functional contributions of CFH and CFHR-mediated signaling to the innate-immune response in inflammatory neurodegeneration are currently not completely understood [36,55,75].

Concluding comments

Recent experimental evidence continues to support the idea that the GI-tract microbiome is capable of providing a rich source of potentially neurotoxic mediators capable of crossing the age-compromised or diseased GI-tract endothelial cell barriers into the systemic circulation and then transit the BBB into the brain parenchyma. Age-related disease or dysfunction of these physiological barriers, poor diet and nutrition in the elderly, or the membrane- and adhesion protein-disruptive properties of the neurotoxins themselves may contribute to leakage of these pathogenic species into the systemic circulation and/or the establishment in the GI-tract microbiome of bacterial dysbiosis that further supports the generation of these neurotoxic components. GI-tract-derived toxins in the systemic circulation such as BF-LPS capable of crossing the BBB have strong potential to trigger the

NF-kB (p50-p65)-miRNA-146a-miRNA-155 signaling system to convey GI-tract microbiome-derived pathogenic signals into the brain. These have strong potential to down-regulate a select number critical mRNAs and their expression, such as for example, brain CFH, to induce disruption of the innate-immune response and inflammatory neurodegeneration (Fig. 1). Other up-regulated miRNAs appear to trigger neurological disease via the targeting and down-regulation of brain-enriched mRNAs, and hence gene expression, involved in the normal maintenance of the cytoskeleton and cytoarchitecture, both pre- and post-synaptic organization, neurotrophic support, amyloidogenesis and the clearance of AD-related lesions such as amyloid peptides [28,34,59,67,83,88]. We speculate that it may be significant that the first anaerobic bacterial species intensively studied as a potential contributor to the onset of AD, that being the Gram negative bacillus *B. fragilis,* appears to utilize damaged or leaky physiological barriers and an activated NF-kB (p50-p65)-pro-inflammatory miRNA-146a-miRNA-155 signaling system to convey microbiome-derived pathogenic signals into the brain [28,34,57,63,85,86,89,90]. Interestingly, the human neurotrophic virus herpes simplex 1 (HSV-1) also activates an NF-kB (p50-p65)-pro-inflammatory miRNA-146a signaling system during the initial infection of human brain neurons, but the nature and significance of this activation pathway, and whether or not this represents an immune evasion strategy by HSV-1 or the host cell, is currently not well understood [24,30,44].

Lastly, the development of strategies for regulating and maintaining a healthy GI-tract microbiome represents a valid, attainable and testable hypothesis for lowering an individual risk and the prevalence of progressive inflammatory neurodegeneration. Indeed, the modification of the GI-tract microbiome composition and complexity via diet-based therapies, prebiotics or probiotics may support: (i) the establishment and maintenance of host-friendly, health-promoting and disease-reducing microbial composition; and (ii) provide new preventive and/or therapeutic options for a more effective clinical management of AD and related forms of age-related inflammatory neurodegeneration [4,5,23,38,40,61,81,89,90].

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Ethics statement

All acquisition, handling, experimental and analytical procedures involving postmortem human brain tissues were carried out in an ethical manner in strict accordance with the ethics review board policies at brain and tissue donor institutions and at the Louisiana State University (LSU) Health Sciences Center. Informed consent from next of kin was obtained at brain and tissue donor institutions for all tissue samples prior to autopsy and donation; coded postmortem brain tissue samples (containing no personal identifying information of the donors) were obtained from the 18 brain and tissue banks listed in the Acknowledgements section above. The ethical use of postmortem human brain tissues and their analyses were also carried out in strict accordance with the Institutional Biosafety Committee and the Institutional Review Board Committee (IBC/IRBC) ethical guidelines IBC#18059 and IRBC#6774 at the LSU Health Sciences Center, New Orleans, LA 70112, USA.

Disclosure

The authors report no conflict of interest.

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