Protective roles of intestinal microbiota derived short chain fatty acids in Alzheimer’s disease-type beta-amyloid neuropathological mechanisms

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To cite this article: Lap Ho, Kenjiro Ono, Mayumi Tsuji, Paolo Mazzola, Risham Singh & Giulio M. Pasinetti (2018) Protective roles of intestinal microbiota derived short chain fatty acids in Alzheimer’s disease-type beta-amyloid neuropathological mechanisms, Expert Review of Neurotherapeutics, 18:1, 83-90, DOI: 10.1080/14737175.2018.1400909

To link to this article: https://doi.org/10.1080/14737175.2018.1400909
ABSTRACT
Background: Dietary fibers are metabolized by gastrointestinal (GI) bacteria into short-chain fatty acids (SCFAs). We investigated the potential role of these SCFAs in β-amyloid (Aβ) mediated pathological processes that play key roles in Alzheimer’s disease (AD) pathogenesis.
Research design and methods: Multiple complementary assays were used to investigate individual SCFAs for their dose-responsive effects in interfering with the assembly of Aβ1-40 and Aβ1-42 peptides into soluble neurotoxic Aβ aggregates.
Results: We found that several select SCFAs are capable of potently inhibiting Aβ aggregations, in vitro.
Conclusion: Our studies support the hypothesis that intestinal microbiota may help protect against AD, in part, by supporting the generation of select SCFAs, which interfere with the formation of toxic soluble Aβ aggregates.

1. Introduction
The term intestinal microbiota refers to the tens of trillions of commensal, symbiotic, and pathogenic microorganisms, including bacteria, fungi, and archaea, which live in our intestine. There is a growing interest in the potential contributions of intestinal microbiota, particularly among the intestinal bacterial population, in human health and/or disease [1]. There is a tremendous diversity among individuals’ intestinal microbiota with respect to the composition of specific bacterial species and the density (number) of bacteria that are present for each of these bacterial species. Indeed, such interpersonal differences in intestinal bacteria composition have been associated with the presence or absence of an increasing number of health issues, including metabolic syndrome, obesity, immunological diseases, cardiovascular diseases, as well as neurodegenerative disorders such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) [1–4].

Gastrointestinal (GI) bacteria may affect host functions by interfering with potential pathogens, improving barrier function, immunomodulation, and/or production of neurotransmitters [5]. GI bacteria may also affect host functions through diet-based microbial influences, by metabolizing dietary compounds into readily absorbable, biologically available, bioactive forms that are responsible for modulation of select biological processes [6]. Accumulating evidence has demonstrated that such diet-based microbial influences may help promote resilience against diverse medical conditions, including neurological disorders such as AD, by modulation of metabolic and immunologic responses and/or other disease-specific pathological mechanisms [7–10]. An example of such diet-based microbial influence on neurodegenerative disorders is the critical role of gut microbiota in the bioactivity of dietary polyphenols. It has been estimated that about 90% of the dietary polyphenols are not absorbed by the small intestine and are accumulated in the colon where they are subjected to metabolism by GI microbial into phenolic acids, which are then readily absorbed [11,12]. Recent evidence revealed that some of these biologically available polyphenolic acids such as caffeic acid and ferulic acid [13–16] are bioactive in inhibiting the generation of beta-amyloid (Aβ) peptides, a key pathogenic feature of AD [8], as well as in suppressing the elevated oxidative stress and inflammatory responses that are observed in AD as well as in other neurodegenerative disorders [9,10]. Moreover, our recent evidence revealed that other biologically available, GI microbiota-derived phenolic acids, such as 3-hydroxybenzoic acid and 3-(3’-hydroxyphenyl)propionic acid, are bioactive in interfering with the misfolding of Aβ peptides into neurotoxic Aβ aggregates that play key roles in AD pathogenesis [17]. Collectively, this evidence suggests that the GI microbiota may help attenuate the development and/or progression of AD through the generation of microbiota-derived phenolic compounds that mechanistically target diverse pathogenic mechanisms underlying AD.

Another example of diet-based microbial influence on AD is the involvement of GI microbials in the metabolism of
20 solutions were prepared and A\(\beta\) peptide in the 1-mM micro-molar ratio of in vitro solutions (0.5-ml aliquots were replaced in 1-mM microcentrifuge tubes. Test compounds (SCFAs) were dissolved in ethanol to a final stock concentration of 2.5 mM. Peptide aggregation was conducted in 10 mM phosphate, pH 7.4, with 5 mM A\(\beta\)–40 (or A\(\beta\)–42) peptide in the presence of either vehicle or individual SCFA a final 5 \(\mu\)M or 20 \(\mu\)M concentration, for a final SCFA:A\(\beta\) molar ratio of 1:1 or 1:4. The tubes were incubated at 37°C for 0–10 days without agitation.

2.4. Peptide aggregation
Aggregation of A\(\beta\)–40 (or A\(\beta\)–42) peptide was conducted essentially as described previously [25]. In brief, A\(\beta\) solutions (0.5-ml aliquots) were placed in 1-ml microcentrifuge tubes. Test compounds (SCFAs) were dissolved in ethanol to a final stock concentration of 2.5 mM. Peptide aggregation was conducted in 10 mM phosphate, pH 7.4, with 5 mM A\(\beta\)–40 (or A\(\beta\)–42) peptide in the presence of either vehicle or individual SCFA a final 5 \(\mu\)M or 20 \(\mu\)M concentration, for a final SCFA:A\(\beta\) molar ratio of 1:1 or 1:4. The tubes were incubated at 37°C for 0–10 days without agitation.

2.5. Photoinduced cross-linking of unmodified proteins
A\(\beta\) peptide oligomer frequency distributions were assessed using the photoinduced cross-linking of unmodified proteins (PICUP) protocol as described previously [25]. Briefly, 1 \(\mu\)l of 1 mM tris(2,2’-bipyridyl)dichlororuthenium(II) (Ru (bpy)) and 1 \(\mu\)l of 20 mM ammonium persulfate were added to 18 \(\mu\)l of freshly prepared protein solution. We noted that addition of SCFA did not lead to observable change in the pH of reaction mixture. The mixture was irradiated for 1 s with visible light, and then the reaction was quenched with 10 \(\mu\)l of Tricine sample buffer (Invitrogen) containing 5% (v/v) \(\beta\)-mercaptoethanol. An aliquot (20 \(\mu\)l) of each cross-linked sample was electrophoresed on a 10–20% gradient Tricine gel and visualized by silver staining (SilverXpress, Invitrogen). Non-cross-linked samples were used as controls in each experiment. To produce intensity profiles and calculate the relative amounts of each oligomer type, Densitometry was performed, and One-Dscan software (v. 2.2.2; BD Biosciences Bioimaging) was used to determine peak areas of baseline corrected data.

2.6. Thioflavin T spectroscopic assay
The thioflavin T (ThT) assay was conducted essentially as described previously [25]. Ten microliter of sample was added to 190 \(\mu\)l of ThT dissolved in 10 mM phosphate buffer, pH 7.4, and then the mixture was vortexed briefly. ThT fluorescence was determined three times at intervals of 10 s using an Hitachi F-4500 fluorometer. Excitation and emission wavelengths were 450 and 482 nm, respectively. Sample fluorescence was determined by averaging the three readings and subtracting the fluorescence of a ThT blank.
2.7. Electron microscopy

To study protofibril formation and the effects of SCFAs on it, Aβ was incubated according to the aggregation protocol above. After incubation at 37°C for 7 days in 10 mM sodium phosphate, pH 7.4, Electron microscopy (EM) was used to determine the morphologies of Aβ1–40 or Aβ1–42 assemblies as described previously [25]. Briefly, a 10-μl aliquot of each sample was spotted onto a glow-discharged, carbon-coated Formvar grid (Electron Microscopy Sciences, Hatfield, PA) and incubated for 20 min. The droplet then was displaced with an equal volume of 2.5% (v/v) glutaraldehyde in water and incubated for an additional 5 min. Finally, the peptide was stained with 8 μl of 1% (v/v) filtered (0.2 μM) uranyl acetate in water (Electron Microscopy Sciences). This solution was wicked off, and then the grid was air dried. Samples were examined using a JEOL CX100 transmission electron microscopy.

3. Results

3.1. Effects of SCFAs on Aβ protein–protein interactions

The self-assembly of Aβ peptides into neurotoxic soluble Aβ aggregates is one of the key neuropathological processes underlying AD. We used the PICUP assay to monitor the effect that individual SCFAs have in interfering with initial protein–protein interactions necessary for the assembly of Aβ1–40 or Aβ1–42 peptides into neurotoxic aggregates in the presence or absence of individual SCFAs at a SCFA:Aβ molar ratio of 1:1 or 1:4. Six SCFAs were tested using the PICUP assay: valeric acid, isovaleric acid, butyric acid, isobutyric acid, propionic acid, and acetic acid. Monomeric Aβ1–40 or Aβ1–42 peptides and cross-linked multimeric Aβ forms were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining.

In the absence of cross-linking, only Aβ1–40 monomers were observed (Figure 1(a,b), lane 1). As we have previously reported [26], cross-linking of Aβ1–40 in the absence of SCFAs leads to the formation of Aβ1-40 dimer and trimer forms (Figure 1(a,b), lane 2, indicated by black arrow). We observed that valeric acid potently interferes with initial Aβ1-40 protein–protein interactions. In particular, the addition of valeric acid at a SCFA:Aβ molar ratio of 1:1 (Figure 1(a,b), lane 13) completely inhibited the formation of the trimeric Aβ1-40 form and partially inhibited the formation of the dimer Aβ1-40 form (compare lane 13 vs. lane 2). The addition of an increasing molar ratio of valeric acid with a SCFA:Aβ molar ratio of 4:1 (Figure 1(a,b), lane 14) completely blocked the formation of into dimeric or trimeric Aβ1-40 forms (compare lane 14 vs. lane 2). Both butyric acid and propionic acid also interfered with Aβ1-40 oligomerization but to a lesser extent in...
comparison to valeric acid. In particular, butyric acid, at a SCFA:Åβ molar ratio of 1:1 (Figure 1(a), lane 11) or 4:1 SCFA:Åβ (Figure 1(a), lane 12), almost completely inhibited the aggregation of Åβ1-40 monomers into Åβ1-40 trimers, but was not effective in modulating the formation of Åβ1-40 dimeric forms (compare lanes 11 and 12 vs. lane 2). Propionic acid, at a SCFA:Åβ ratio of 4:1 (Figure 1(a), lane 10), almost completely inhibited the generation of Åβ1-40 trimers (compare lane 10 vs. lane 2). However, Åβ1-40 aggregation was not affected by the addition of a lower molar ratio of propionic acid, at a 1:1 SCFA:Åβ molar ratio (Figure 1(a), lane 9) (compare lane 9 vs. lane 2). The remaining three SCFAs monitored (isobutyric acid, isovaleric acid, and acetic acid) had no observable impact on the conversion of monomeric Åβ1-40 peptide (Figure 1(a), lanes 3–8) into higher-ordered multimeric Åβ1-40-forms in our PICUP assay (compare lanes 3–8 vs. lane 2).

In the absence of cross-linking, monomeric Åβ1-42 shows up in a trimeric form under SDS-PAGE (Figure 1(c,d), lane 1). The Åβ1-42 trimer band has been shown to be an SDS-induced artifact [26,27]. As we have previously reported [26], cross-linking of Åβ1-42 in the absence of SCFAs leads to the formation of Åβ1-42 oligomers of orders 2–6 (Figure 1(c,d), lane 2, indicated by black arrow). We observed that the addition of valeric acid, at a SCFA:Åβ molar ratio of 4:1 (Figure 1(c,d), lane 14), completely inhibited the formation of all Åβ1-42 oligomers (compare lane 14 vs. lane 2). However, Åβ1-42 aggregation was not affected by the addition of a lower molar ratio of valeric acid, at a 1:1 SCFA:Åβ molar ratio (Figure 1(c,d), compare lane 13 vs. 2). The conversion of Åβ1-42 monomers into higher-order multimeric forms was unaffected by the addition of butyric acid, propionic acid, isobutyric acid, isovaleric acid, and acetic acid at either a SCFA:Åβ molar ratio of 1:1 or 4:1 (Figure 1(c,d), compare lanes 2–12 vs. lane 2).

In parallel, in the control PICUP study, which used GST as a positive control for the cross-linking chemistry as we have used in the past [25], we observed that incubation of GST in the absence of SCFAs leads to the formation of higher molecular weight GST aggregate forms (Figure 1(e,f), lane 2, indicated by a black arrow). We observed no alterations in GST cross-linking in the presence of SCFAs at a SCFA:GST molar ratio of 1:1 (Figure 1(e,f)), compare lanes 3, 5, 7, 8, 9, 11, 13 vs. lane 2) or 4:1 (Figure 1(e,f), compare lanes 4, 6, 8, 10, 12, 14 vs. lane 2). We therefore concluded that valeric acid, butyric acid, and propionic acid inhibited Åβ1-40 and/or Åβ1-42 aggregation.

3.2. Effects of SCFAs on Åβ fibril formation

Based on the evidence from our PICUP assay, we continued by assessing valeric acid and butyric acid for their potential effects on the assembly of monomeric Åβ peptides into Åβ fibrils using the ThT spectroscopic assay to monitor for temporal changes in β-sheet contents of Åβ1-40 and Åβ1-42, which were incubated in the absence of SCFA (SCFAS:Åβ molar ratio of 0:1) or in the presence of individual SCFAs at a SCFA:Åβ molar ratio of 1:1 or 4:1. We note that ThT fluorescence is not a direct measure of fibril content. However, since β-sheet formation correlates with fibril formation, ThT fluorescence is a useful surrogate marker [28].

We observed that both valeric acid and butyric acid attenuated the conversion of Åβ1-40 monomers to Åβ1-40 fibrils with a dose-response efficacy; the higher dose of valeric acid or butyric acid (SCFA:Åβ molar ratio of 4:1) appeared more effective than the lower dose (SCFA:Åβ molar ratio of 4:1) (Figure 2(a,b)). We found that valeric acid also attenuated the conversion of Åβ1-42 monomers to Åβ1-42 fibrils with a dose-response efficacy; the higher dose of valeric acid (SCFA:Åβ molar ratio of 4:1) appeared more effective than the lower dose (SCFA:Åβ molar ratio of 4:1) (Figure 2(c)). Lastly, we observed that butyric acid treatment also displayed a tendency to reduce the formation of Åβ1-42 fibrils, but there are no observable differences between the lower and higher dose (Figure 2(d)).

Collectively, our evidence demonstrates that valeric acid (and to a lesser extent, butyric acid) is capable of interfering with the conversion of monomeric Åβ1-40 and Åβ1-42 into Åβ fibrils.

3.3. Effects of valeric acid on the morphologies of the Åβ assemblies

Our evidence from Figures 1 and 2 demonstrating that, among the six SCFAs we tested, valeric acid most potently interferes with the aggregation of Åβ peptides into higher-ordered assemblies. Based on this, we continued and used EM to monitor for effects of valeric acid on the morphologies of the Åβ1-40 and Åβ1-42 assemblies. Consistent with our prior observations [25], the incubation of Åβ1-40 (Figure 3, panel I) or Åβ1-42 (Figure 3, panel III) in the presence of vehicle leads to the generation of classical non-branched fibrils with helicity features. Åβ incubation in the presence of valeric acid, at a SCFA:Åβ molar ratio of 4:1, almost completely inhibited fibril formation from Åβ1-40 (Figure 3, panel II) or Åβ1-42 (Figure 3, panel IV).

4. Discussion

The assembly of Åβ peptides into low-n oligomers requires initial protein–protein interactions among Åβ peptides. Our study is specifically designed to assess if a specific test compound is capable of interfering with such protein–protein interactions and therefore to disrupt the process Åβ assembly into low n neurotoxic oligomers. We used independent in vitro assays to investigate six SCFAs that are derived from GI microbiota metabolism of dietary fibers for their potential effects in modulating the assembly of Åβ1-40 and Åβ1-42 peptides to soluble, neurotoxic Åβ aggregates that play roles in AD pathogenesis. Results from our PICUP assays revealed that select SCFAs, particularly valeric acid, butyric acid, and propionic acid are capable of interfering with initial protein–protein interactions,
which are necessary for A\(\beta\) assemblies (Figure 1). In particular, valeric acid, butyric acid, and propionic acid demonstrated efficacy in interfering with protein–protein interactions that are necessary for the conversion of A\(\beta\) peptides to neurotoxic A\(\beta\) aggregates. The relative anti-A\(\beta\) aggregation efficacy of the three SCFAs is, in decreasing order, valeric acid \(\gg\) butyric acid \(\gg\) propionic acid. In particular, valeric acid most potently inhibited protein–protein interaction of A\(\beta\)1-40 and A\(\beta\)1-42; the higher dose of valeric acid (at a SCFA:A\(\beta\) molar ratio of 4:1) interfering with the aggregation of both A\(\beta\)1-40 and 1-42 peptides. In comparison, the lower dose (at a valeric acid:A\(\beta\) molar ratio of 1:1) was effective in interfering with the aggregation A\(\beta\)1-40 into A\(\beta\)1-40 trimers, and to a lesser extent A\(\beta\)1-40 dimers, but was not effective in interfering with the aggregation of A\(\beta\)1-42. In contrast, butyric acid (at both the higher and lower dose) and propionic acid (at the higher dose) interfered with the formation of A\(\beta\)1-40 trimers, but not the formation of A\(\beta\)1-40 dimers. Moreover, either butyric acid or propionic acid was effective in modulating the aggregation of A\(\beta\)1-40 peptides into higher-order aggregate forms. In contrast, three of the SCFAs tested (isobutyric acid, isovaleric acid, and acetic acid) had no observable effects on A\(\beta\) assemblies, as assessed by the PICUP assay. The efficacy of valeric acid and butyric acid in interfering with the assemblies of higher-ordered A\(\beta\) aggregate forms detectable by the PICUP assay is validated by observations from the independent ThT assay (Figure 2). Consistent with results from our PICUP assays, we observed that valeric acid and butyric acid were effective in interfering with the assembly of A\(\beta\)1-40, and to a lesser extent, the assembly of A\(\beta\)1-42 into A\(\beta\) fibrils. The efficacy of valeric acid in interfering with the generation of A\(\beta\) fibrils was validated by the EM assay, which demonstrated that valeric acid inhibited the assembly of A\(\beta\)1-40 or A\(\beta\)1-42 into A\(\beta\) fibrils with classical non-branched fibrils with helicity features (Figure 3). Collectively, results from our studies support the hypothesis that intestinal microbiota may have protective effects against AD, in part, by supporting the generation of select SCFAs that interfere with the formation of toxic soluble A\(\beta\) aggregates. While the microbiota is known for generating SCFAs for dietary fibers, we also note that relatively minor amount of SCFA can be of dietary nature. Thus it is possible that dietary SCFA may also

Figure 2. Select SCFAs potently interfere with A\(\beta\) fibril formation. Assembly of monomeric A\(\beta\)1-40 or A\(\beta\)1-42 peptides into A\(\beta\) fibrils in the presence of valeric acid, butyric acid or vehicle were assessed using the ThT assay, which monitors ThT fluorescence as an indirect assessment of fibril contents. Periodically, aliquots were removed, and ThT binding levels were determined. Binding is expressed as mean fluorescence (in arbitrary fluorescence units (FU)). (a-d) A\(\beta\)1-40 (a-b) or A\(\beta\)1-42 (c-d) were incubated for up to 240 min at 37°C in 10 mM phosphate, pH 7.4, in the presence of vehicle (l), or in the presence of valeric acid (a,c) or butyric acid (b,d) at a SCFAs:A\(\beta\) molar ratio of 1:1 (l) or 4:1 (l). Shown are representative assays from three independent studies.
modulate Aβ aggregation independent of contribution from GI microbiota.

The misfolding of disease-specific proteins into toxic aggregates is an important and common pathological mechanism underlying diverse neurological disorders. Aside from misfolding of Aβ peptides in AD, there are many other neurological disorders in which protein misfolding plays a crucial role in disease pathology, such as misfolding of α-synuclein in α-synucleinopathies such as PD, dementia with Lewy bodies, and multiple system atrophy, and misfolding of tau in tauopathies such as AD, dementia pugilistica, progressive supranuclear palsy, among others [29–32]. These aggregating proteins all share similar biophysical and biochemical properties that influence how they misfold, aggregate, and propagate in disease [33]. Thus, in addition to interfering with abnormal aggregation of amyloidogenic Aβ isoforms, valeric acid, butyric acid, and propionic acid may also similarly interfere with the assembly of α-synuclein and tau.

Accumulating published evidence suggests that GI bacteria may help promote resilience against AD through multiple mechanisms, the promotion of brain energy metabolism, modulation of neuro-inflammation, and modulation of epigenetic mechanisms (see Figure 4). Our evidence revealed that GI bacteria may also improve AD by supporting the generation of select SCFAs that are capable of interfering with the assembly of Aβ1-40 and Aβ1-42 peptides into neurotoxic Aβ aggregates (see Figure 4). Future in vivo studies will be necessary to clarify whether certain SCFAs may attenuate AD β-amyloidosis through additional mechanisms.

5. Conclusion
Our observations provide the impetus for new investigations to identify and characterize select dietary fibers that support the generation of valeric acid, butyric acid, and propionic acid, as well as GI microbial that are capable of metabolizing dietary fibers to the these SCFAs. Information gathered will lead to the development of next-generation probiotics that might help promote resilience to diverse neurodegenerative disorders.
Key issues

- The studies suggest that select bacterial species that support conversion of dietary fibers to short-chain fatty acids, which are relevant to neurodegenerative conditions.
- Our observation links gastrointestinal microbiota with mechanisms underlying AD-type Aβ neuropathological mechanisms.
- Our observations suggest the feasibility of developing valeric acid for treating AD.
- Misfolding of diverse proteins in multiple neurological disorders, such as Aβ in Alzheimer’s disease, tau in tauopathies, α-synuclein in Parkinson’s disease and Huntington’s disease, all share common mechanistic features. Our observation supports the potential development of valeric acid for treating these diverse neurological disorders.
- Our observations support further investigations to identify and characterize gastrointestinal bacterial specie(s) that support the generation of valeric acid.
- Our observations also support further investigations to characterize select dietary fiber preparations that supports the generation of valeric acids.
- Information from the study will lead to the development of next-generation probiotics that might help promote resilience to diverse neurodegenerative disorders.

Funding

This study was supported in part by Grant Number P50 AT008661-01 from the NCCIH and the ODS. GM Pasinetti holds a VA Career Scientist Award. The authors acknowledge that the contents of this study do not represent the views of the NCCIH, the ODS, the NIH, the U.S. Department of Veterans Affairs, or the United States Government.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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