ORIGINAL ARTICLE

Angiotensin (1–7) delivered orally via probiotic, but not subcutaneously, benefits the gut-brain axis in older rats

Thomas W. Buford · Yi Sun · Lisa M. Roberts · Anisha Banerjee · Sujitha Peramsetty · Anthony Knighton · Amrisha Verma · Drake Morgan · Gonzalo E. Torres · Qiuhong Li · Christy S. Carter

Abstract To (1) investigate the efficacy of multiple doses of an orally delivered probiotic bacteria *Lactobacillus paracasei* (LP) modified to express angiotensin (1–7) (LP-A) in altering physiologic parameters relevant to the gut-brain axis in older rats and to (2) compare this strategy with subcutaneous delivery of synthetic Ang(1–7) peptide on circulating Ang(1–7) concentrations and these gut-brain axis parameters. Male 24-month-old F344BN rats received oral gavage of LP-A, or subcutaneous injection of Ang(1–7) for 0×, 1×, 3×, or 7×/week over 4 weeks. Circulating RAS analytes, inflammatory cytokines, and tryptophan and its downstream metabolites were measured by ELISA, electrochemiluminescence, and LC-MS respectively. Microbiome taxonomic analysis of fecal samples was performed via 16S-based PCR. Inflammatory and tryptophan-related mRNA expression was measured in colon and pre-

frontal cortex. All dosing regimens of LP-A induced beneficial changes in fecal microbiome including overall microbiota community structure and α-diversity, while the 3×/week also significantly increased expression of the anti-inflammatory species *Akkermansia muciniphila*. The 3×/week also increased serum serotonin and the neuroprotective analyte 2-picolinic acid. In the colon, LP-A increased quinolinate phosphoribosyltransferase expression (1×/week) and increased kynurenine aminotransferase II (1× and 3×/week) mRNA expression. LP-A also significantly reduced neuro-inflammatory gene expression in the prefrontal cortex (3×/week: COX2, IL-1β, and TNFα; 7×/week: COX2 and IL-1β). Subcutaneous delivery of Ang(1–7) increased circulating Ang(1–7) and reduced angiotensin II, but most gut-brain parameters were unchanged in response. Oral—but not subcutaneous—

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Ang(1–7) altered physiologic parameters related to gut-brain axis, with the most effects observed in 3×/week oral dosing regimen in older rats.

Introduction

Advanced age is associated with a progressive decline in various physiologic functions which predispose older adults to a host of clinical disorders and diseases. Late-life cognitive decline—whether mild cognitive impairment or that attributable to Alzheimer's disease or related dementias—represents one of the most substantial aging-related public health concerns. Despite extensive exploration into underlying mechanisms and potential treatments for age-related cognitive declines, efficacious therapy remains elusive. Thus, novel thinking and new strategies are needed to preserve cognitive function among a large segment of the rapidly expanding older adult population.

In recent years, the role of the intestinal tract (i.e., the "gut") has received increased attention for its role in heath and various physiologic processes—including neural communication and cognitive function via an established "gut-brain axis" (Collins et al. 2012; Kennedy et al. 2017; Strandwitz 2018). Notably, advanced age is associated with changes in the gut microbiome that affect both the density and composition of microbiota (i.e., dysbiosis),(Biagi et al. 2010; Jeffery et al. 2016) and these changes have been associated with the development of cognitive impairment (Cattaneo et al. 2017; Lawrence and Hyde 2017). Thus, the gut may be a promising target for intervention to preserve cognitive functions in late life.

One method of addressing age-related dysbiosis is through dietary supplementation with probiotics. Probiotics provide a variety of beneficial effects including antimicrobial activity, enhancement of intestinal barrier function, and immunomodulation via actions on a wide variety of immune cells (Ng et al. 2009). Consistent with these established mechanisms, several studies among older adults have demonstrated beneficial effects of probiotic preparations on gut microflora composition (Ahmed et al. 2007; Rampelli et al. 2013; Liu et al. 2016) and systemic immunity (Gill et al. 2001; Moro-Garcia et al. 2013; Liu et al. 2016). For example,

probiotics have been proposed as a safe and efficacious method to deliver other therapeutic compounds as genetically modified probiotics (GMPs) offer a potentially efficacious method to deliver drugs or other therapeutic proteins with precision and a higher degree of site specificity than convention drug regimens (Steidler 2003; Syvanen 2003; Paton et al. 2012; M. Kumar et al. 2016).

The primary objective of the work presented here was to evaluate the efficacy of differing doses of a GMP designed to deliver the bioactive peptide angiotensin (1–7) (Ang[1–7]) on physiologic parameters related to the gut-brain axis. Ang(1–7) is a key vasoprotective effector of the renin-angiotensin system (RAS) that opposes the fibrotic, vasoconstrictive, proliferative, and inflammatory effects of angiotensin 2 (AngII). Components of the Ang(1–7)/Mas axis are found throughout the central nervous system (Young et al. 1988; Chappell et al. 1989; Hamming et al. 2004; Gallagher et al. 2006; Doobay et al. 2007; Becker et al. 2007; Guo et al. 2010) and several studies indicate that Ang(1–7) benefits various aspects of cognition in rodents (Xie et al. 2014; X. L. Wang et al. 2016a; L. Wang et al. 2016b; Lazaroni et al. 2016; Kangussu et al. 2017). More recent evidence also suggests a specific benefit on AD pathobiology, including mitigation of tau deposition (Jiang et al. 2016a; Jiang et al. 2016b; Kehoe et al. 2016).

We previously reported that oral delivery *Lactobacillus paracasei* expressing Ang(1–7) (LP-A) significantly increased circulating concentrations of Ang(1–7) in older Fischer 344/Brown-Norway (F344/BN) rats (Carter et al. 2019)—demonstrating efficacy of the compound in surviving the gut. Here, we investigate the efficacy of multiple doses of LP-A in altering several physiologic parameters relevant to the gut-brain axis including gut microbiome composition, systemic metabolites of tryptophan involved in neurotransmission, and neuro-inflammatory gene expression within the pre-frontal cortex, a brain region underlying multiple cognitive functions that decline with age. Moreover, a secondary objective of this work was to evaluate the impact of varying doses of subcutaneous, in contrast to oral Ang-(1–7) expressed from probiotic bacteria, delivery of Ang(1–7) on circulating Ang(1–7) concentrations and these gut-brain axis parameters.

Methods

Experimental overview

This work outlines findings from two complimentary 4-week dosing studies designed to (1) identify the optimal dose of orally delivered LP-A for inducing changes in several physiologic indices relevant to the gut-brain axis and to (2) evaluate the efficacy of subcutaneous Ang(1–7) administration for increasing systemic concentrations of relevant RAS analytes, including Ang(1–7), and in altering the same indices of gut-brain physiology. Studies were completed at the University of Florida and the University of Alabama at Birmingham, with all experimental protocols approved by the respective Animal Care and Use Committees in accordance with the "Guide for the Care and Use of Laboratory Animals." All animals were obtained from the National Institute on Aging (NIA) Colony at Harlan Laboratories (Indianapolis, IN). Due to ongoing shortages of female F334/BN rats in the colony, all experiments were completed in male rats with subsequent experiments planned once females become available.

Animals

Male F344BN rats (oral study $N = 30$; subcutaneous study $N = 40$) were obtained from the NIA colony at 24 months of age and individually housed on a 12-h light and 12-h dark cycle in a specific pathogen-free facility accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were fed a standard rodent chow (18% kcal from fat, no sucrose, 3.1 kcal/g, diet 2018; Harlan Teklad, Madison, WI). Animals were allotted 1 week to acclimate to their housing conditions and to establish baseline rates of food intake and body weight. Health status, body weight, and food intake were monitored daily. Health assessments included checking for a sudden decline in body weight, redness around the eyes and nostrils, ruffled coat, open tail sores, and haunched posture.

Experimental design

Oral study Animals ($n = 6–8$/group) were randomized at 24 months of age to the following treatment groups for 4 weeks: vehicle (trehalose, ascorbic acid, and skim milk) or *Lactobacillus paracasei* expressing Ang-1-7 (LP-A) 1×, 3×, or 7×/week as described (Carter et al.

2019). The concentration of the LP-A did not change, but rather the number of days/week the LP-A was delivered. On days when rats in the 1× and 3×/week group did not receive the LP-A, they were gavaged with vehicle to control for the stress of being handling and gavaged to a similar degree as the 7×/week group. Animals were weighed and their food intake measured daily to ensure there were no anorectic or other adverse effects of the drug administration.

Subcutaneous study Animals ($n = 10$/group) were randomized at 24 months of age to the following treatment groups for 4 weeks: PBS vehicle or Ang(1–7) for 1×, 3×, or 7×/week. Again, the concentration of the Ang(1–7) did not change but rather the number of days/week the Ang(1–7) was delivered via subcutaneous injection. On days when rats in the 1× and 3×/week group did not receive the Ang(1–7), they were injected with PBS to control for the stress of being handling and injected to a similar degree as the 7×/week group. PBS group received injection 7×/week. Animals were weighed daily to ensure there were no anorectic or other adverse effects of the drug administration. One animal died of unknown causes at day 22 in the Ang(1–7) 3× group; therefore, this group had $n = 9$.

Probiotic administration

For the oral study, LP-A were cultured in MRS (deMan Rogosa Sharpe) broth (BD Difco, Houston, TX, USA) supplemented with 5 µg/ml erythromycin at 37 °C for 18 h. The bacteria were harvested by centrifugation at 5000×g for 20 min and resuspended in sterile PBS for oral gavage. For extended storage, harvested bacteria were washed once with PBS and then suspended in TAS buffer (4% trehalose, 4% sodium ascorbate, and 6% skim milk) and frozen in small aliquots at − 20 °C. Colony counting was conducted before the animal experiments to ensure the numbers of surviving bacteria. Animals were orally gavaged with 2×10^{11} CFU/kg body weight or equal volume of buffer. An 18-gauge gavage/feeding needle (3-in. length/3-mm ball diameter) was inserted into the esophagus, ensuring that there was no resistance to its advancement. The fluid was injected slowly and when complete, the needle was pulled straight out. The rats were tolerant of this procedure and no averse outcomes were observed over the 4-week study.

Subcutaneous Ang(1–7) administration

For the subcutaneous study, animals were subcutaneously injected with 1 mg/kg body weight of Ang(1–7) (TXA 127, Constant Therapeutics, Boston, MA) or an equal total injection volume of PBS, ranging between 1.8 and 2.2 ml. The rat was manually restrained and then placed on a solid surface. The skin of the back area was tented by the thumb and forefinger. A 23-gauge needle (BD, Franklin Lakes, NJ, USA) connected to a 3-ml syringe (BD, Franklin Lakes, NJ, USA) was inserted under the lifted skin. The fluid was injected slowly and when complete, the needle was pulled straight out. The rats were tolerant of this procedure and no averse outcomes were observed over the 4-week study.

Tissue collection and euthanasia

On day 8, blood was collected from the tongue of an anesthetized (5% isoflurane) rat that was grasped with tweezers, the sublingual vein punctured with a ¾-in., 26-gauge needle, and blood allowed to drip into a serum tube (BD, Franklin Lakes, NJ, USA). A cotton swab was used to stop bleeding. Rats recover, within 5 min, in their home cages with a warming disk placed underneath the cage. On day 29, rats were euthanized by rapid decapitation with a guillotine (Harvard Apparatus, Holliston, MA), and trunk blood was collected directly into a serum collection tube (BD, Franklin Lakes, NJ, USA). Both on day 8 and day 29, blood collected in the serum collection tube was left at room temperature for 30 min and then spun in a centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C at 2000 rpm for 10 min. Supernatant was collected after centrifugation and stored in − 80 °C until analysis.

At the time of euthanasia on day 29, brain (prefrontal cortex) and intestinal (colon) tissues were also collected and immediately removed on ice, snap-frozen in liquid nitrogen, and stored at − 80 °C until analysis. Fecal samples were collected directly from the colon, stored in commercial available preservative Para-Pak (Meridian Bioscience Inc., Cincinnati, OH), flash frozen, and stored at − 80 °C until analysis.

Outcomes

Circulating RAS analytes Circulating concentrations of RAS analytes Ang(1–7), Ang II, angiotensin-converting enzyme (ACE), and ACE2 were measured for the subcutaneous study using commercial assays (Ang[1–7], Ang II) and assays using fluorescent substrates (ACE, ACE2) as previously reported for the oral study (Carter et al. 2019).

Serum cytokines Circulating concentrations (day 29) of interleukins (ILs) 4, 6, and 10 as well as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) were determined using a Meso Scale Discovery (MSD; Rockville, MD) Rat Proinflammatory Panel 2 and a Quick Plex SQ 120 imager using electrochemiluminescence technology. Minimum sensitivity (pg/ml) and intra-assay coefficients of variation (%) for each assay were as follows: IL-4 (0.44, 9.12), IL-6 (25.10, 7.06), IL-10 (3.41, 5.65), IFNγ (6.88, 8.92), TNFα (1.17, 7.21).

Serum tryptophan metabolites Serum concentrations of circulating metabolites reflective of the kynurenine, serotonin, and tryptamine/indole pathways of tryptophan metabolism were determined. Fifty microliters of serum was mixed with 25 ng/ml methanolic tryptophan-d5 internal standard, and 500 μl of acetonitrile 1.0% FA was added to Phree cartridges (Phenomenex, Torrance, CA) on a SPE vacuum manifold. Serum samples were forcefully expelled into acetonitrile contained in Phree cartridge to ensure proper mixing. The mixture was incubated at RT for 5 min. Vacuum was applied to draw the mixture through the sorbent into a borosilicate collection tube. Samples were dried under N2 gas and then reconstituted in 100 μl 0.1% FA.

The LC-MS method used was previously described by Zhu et al. with minor alterations (Zhu et al. 2011). Tryptophan-d5 was the only internal standard employed during this analysis. A shorter Atlantis T3 3 μm 100 × 2.1 mm column (Waters, Milford, MA) was used and a diversion valve was employed to divert column eluent to waste for the first minute of the gradient separation. MultiQuant 3.0.3 was employed for post-acquisition data analysis. All standard curves were linear with $1/x^2$ weighting.

Fecal microbiome taxonomy Taxonomic analysis of the fecal microbiome (day 29) was performed via 16S-based polymerase chain reaction (PCR) procedures as previously described (Buford et al. 2018). Briefly, DNA was extracted from samples and PCR was used with unique bar-coded primers to amplify the V4 region of the 16S rRNA gene to create an "amplicon library" from

individual samples as described by Kumar et al. (R. Kumar et al. 2014). The entire PCR reaction was electrophoresed, the PCR product was visualized by UV illumination, and the band was excised and purified from the agarose.

The PCR products were sequenced and paired-end reads of approximately 250 bp from the V4 region of 16S rDNA were analyzed. The samples were first quantitated using Pico Green, adjusted to a concentration of 4 nM then used for sequencing. Fastq conversion of the raw data files was performed following de-multiplexing, and quality control of the fastq files was performed which was then subject to quality assessment and filtering. One sample was removed from analysis due to failing quality control procedures.

Following quality control procedures, sequences were grouped into operational taxonomic units (OTUs) and taxonomic identification and abundance information was obtained. OTUs were then grouped together to summarize taxon abundance at different hierarchical levels of classification (e.g., phylum, class). Alpha diversity was calculated using Shannon's diversity matrix and beta diversity was measured using unweighted Unifrac analysis. Principal coordinates analysis (PCoA) was performed to visualize the dissimilarity matrix between all samples.

mRNA expression—colon and pre-frontal cortex Colon and pre-frontal cortex samples (striatal tissue dissected from a 3-mm coronal section) of brain tissue were collected, snap-frozen, and kept at − 80 °C until processing for evaluation of gene expression related to tryptophan metabolism (colon) or neuro-inflammation (pre-frontal cortex). Total RNA was isolated from with RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions or via Trizol (Invitrogen, Carlsbad, CA)/alcohol purification (Buford et al. 2009a; Buford et al. 2009b) with further purification via the RNeasy Plus kit (Qiagen). RNA concentration and purity was assessed photometrically using A260/A280 and A260/A230 ratios. RNA was reverse-transcribed to cDNA using the SuperScript VILO cDNA Synthesis kit (Invitrogen, Carlsbad, CA) or High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems), according to manufacturer instructions. The cDNA was stored at − 20 °C until gene expression analysis. Gene expression analysis was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems, Carlsbad, CA) following the

manufacturer's instructions using the StepOne Real-Time PCR System (Applied Biosystems) or via the SYBRgreen master mix using the Rotor-Gene Q PCR (Qiagen). The threshold cycle (C_t) values were determined and analyzed via respective manufacturer software. All samples were examined in triplicate, and genes of interest (Table 1) were expressed relative to the housekeeping gene (β-actin) as fold change from the reference group by the $2^{-\Delta\Delta Ct}$ method. The housekeeping gene expression was not different among groups (data not shown).

Statistics

All data were evaluated for normality and homogeneity of variance prior to determination of descriptive statistics and comparative analyses. When assumptions were met, dependent outcomes other than the 16S microbiome analysis were analyzed using univariate analysis of variance (ANOVA) with pre-planned contrasts comparing each dosing condition (1×, 3×, and 7×) with the 0×/week "control" condition. When assumptions were violated (e.g., non-normal distributions), non-parametric tests were performed. A p value < 0.05 was considered statistically significant. For microbiome data, measures of α-diversity were evaluated as described for other outcomes, while β-diversity differ-

Table 1 Primers for mRNA expression analyses

Gene	Primer
Housekeeping	
Actb (beta-actin)	Rn00667869_m1
Pre-frontal cortex	
Il1a	Rn00566700_m1
Il1b	Rn00580432_m1
Il6	Rn01410330_m1
Ptgs2 (COX2)	Rn01483828_m1
LOC103694380 (TNFα)	Rn99999017_m1
Colon	
Ccbl1 (KAT1)	Rn01439192_m1
Aadat (KAT2)	Rn00567882_m1
Ccbl2 (KAT3)	Rn01522586_m1
Got2 (KAT4)	Rn00820736_g1
Naprt1	Rn01506019_g1
Qprt	Rn01506918_g1
Haao (3-HAO)	Rn01469327_m1

All primers obtained from Applied Biosystems (Carlsbad, CA)

ences were determined via PERMANOVA. Differences between groups were evaluated at each level of phylogeny for OTUs with ≥ 0.5% abundance. A *p* value of < 0.05 was utilized to initially identify OTUs of interest with final determination of significance established after correcting for false discovery rate (FDR) according to the method of Benjamini and Hochberg (1995).

Results

Circulating RAS analytes–subcutaneous administration Subcutaneous administration of Ang(1–7) in varying dosing frequencies altered systemic concentrations of various RAS analytes (Fig. 1).

We have previously reported similar changes in the oral study, including increases after 8 days of delivery that persisted after 29 days of delivery (Carter et al. 2019). Most notably, both the 1×/week ($p = 0.001$) and 3×/week ($p = 0.001$) dosing conditions in the systemic study increased circulating Ang(1–7) at day 29 compared with the control condition, while the Ang(1–7)/AngII ratio was increased in all three dosing conditions compared with control (all *p*'s ≤ 0.001 at 29).

Serum cytokines Circulating cytokine concentrations at day 29 of the oral LP-A delivery experiment are shown in Fig. 2. Modest, but statistically significant differences from the 0×/week dosing group were observed for IL-6 in the 7×/week group ($p = 0.026$) and for TNFα in the 1×/week ($p = 0.037$) and 7×/week ($p = 0.037$) dosing groups. No significant differences were observed for these circulating cytokines in the subcutaneous delivery experiment (Supplemental Fig. 1).

Fecal microbiome analyses Oral LP-A delivery induced several changes in the fecal microbiome of the rats, particularly in the 3×/week group (Fig. 3). Principal coordinates analysis (PCoA) revealed that groups differed in the overall microbiome community structure as determined by unweighted UniFrac ($p = 0.004$) (Fig. 3A), with the most striking difference observed when comparing control (0×/week) with experimental conditions (Fig. 3B). Figure 3 C depicts the overall composition of the fecal microbiomes at the phylum level of taxonomy across dosing groups. Across all groups, the composition of the phylum-level fecal microbiome was 64.9% Firmicutes, 25.1% Bacteroidetes, 4.5% Verrucomicrobia, 2.5% Tenericutes, 1.3%
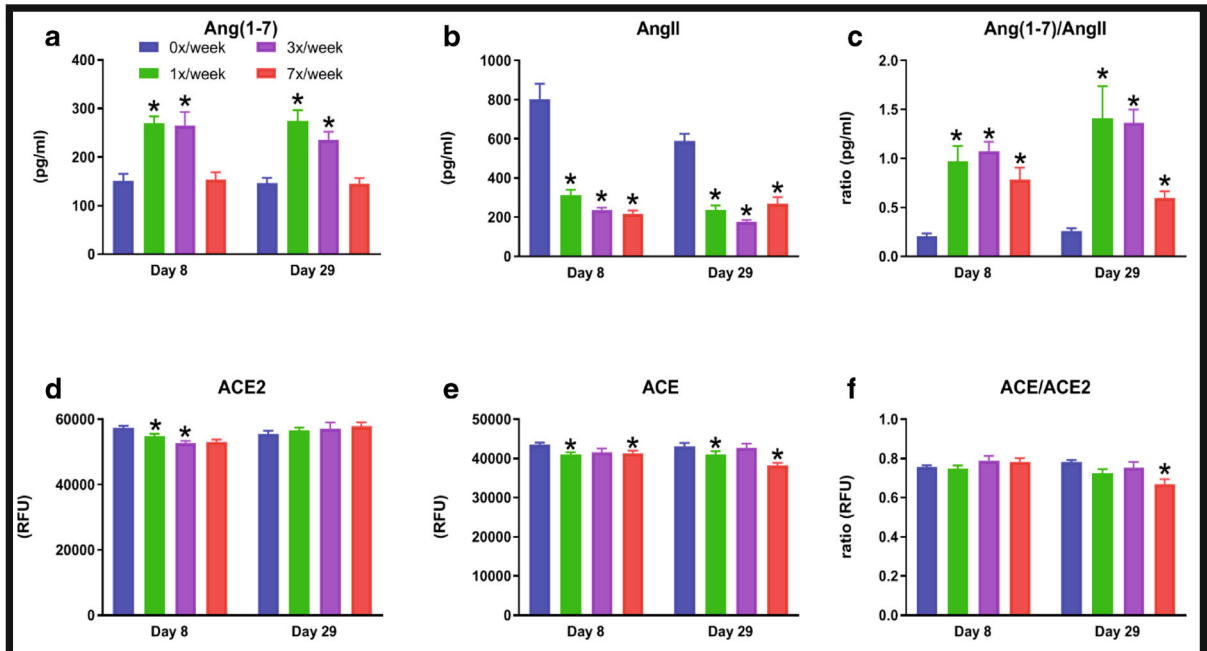

Fig. 1 Circulating RAS analytes were measured in 24-month-old male F344/BN rats after 8 days (acute) and 29 days (chronic) administration of 1 mg/kg of subcutaneous Ang(1–7) in 4 dosing conditions: 0×, 1×, 3×, or 7×/week. The graphs describe results for (A) Ang(1–7); (B) AngII; (C) Ang(1–7)/Ang (II) ratio; (D) ACE2; (E) ACE; and (F) ACE2/ACE ratio. Data are presented as mean ± SD. Asterisks denote $p < 0.05$ from the 0×/week condition at respective time point

Fig. 2 Serum cytokine concentrations among 24-month-old F344/BN rats following 4 weeks of supplementation with oral Ang(1–7) expressing *Lactobacillus paracasei* either 0×, 1×, 3×, or 7×/week. Data are presented as mean ± SD. *$p < 0.05$ from the 0×/week condition
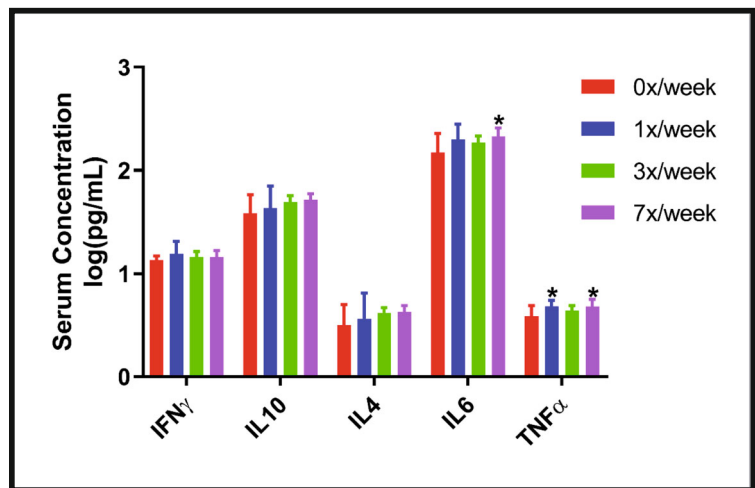
Proteobacteria, 1% Cyanobacteria, and 0.7% from remaining phyla. Key measures of α-diversity, including richness (chao1 and observed species) and phylogenetic diversity, displayed significant group differences—with the most dramatic effects observed in the 3×/week group (Fig. 3D, E, F). Overall sample diversity, measured according to the Shannon and Simpson metrics, did not significantly differ among dosing regimens.

The phyla Verrucomicrobia was significantly enriched by the 3×/week dosing regimen ($p = 0.008$). The 1×/week dosing group had an initial p value < 0.05 for a decline in Proteobacteria ($p = 0.042$) compared with the 0×/week regimen, but this value did not remain significant after FDR correction. At the species level, two bacteria displayed differences in DNA abundance among treatment groups. The bacteria species used for LP-A, *Lactobacillus paracasei*, was significantly enriched among all treatment groups (Fig. 3G) and *Akkermansia muciniphila* (Verrucomicrobia) was enriched in the 3×/week treatment group.

For the subcutaneous study, there were no statistical differences at any level of taxonomy following correction for multiple comparisons (Supplemental Figure 2). Indices of beta and alpha diversity were also not significant, though a trend toward significance was observed for Simpson diversity ($p = 0.068$) for the 7×/week delivery (0.85 ± 0.12) compared with the control condition (0.910 ± 0.05).

Serum tryptophan metabolism Changes observed in the oral study for circulating concentrations of serotonin and kynurenine-related tryptophan metabolites are shown in Fig. 4. Statistically significant differences

from the 0×/week group were observed in the 3×/week cohort for both serotonin ($p = 0.034$) and 2-picolinic acid ($p = 0.045$). No significant differences were observed for tryptamine, indole-3 lactate, or indole-3 acetate (not shown).

In the subcutaneous study, significant differences were observed for serum kynurenine, with concentrations (normalized as ratio to total tryptophan) increased in the 1× (0.70 ± 0.10; $p = 0.009$) and 7×/week (0.67 ± 0.15; 0.034) condition compared with the 0×/week control (0.56 ± 0.11) (Supplemental Fig. 3). Serum serotonin was also significantly increased in the 7×/week condition (0.22 ± 0.15 vs. 0.20 ± 0.16 in 0×/week; $p = 0.035$). No other statistically significant differences were observed in the subcutaneous study.

Colon tryptophan-related mRNA expression In the oral study, we observed significantly lower mRNA expression of quinolinate phosphoribosyltransferase (QPRT; $p = 0.040$) in the 1×/week condition ($2^{-\Delta\Delta CT} = 0.17 \pm 0.20$) compared with 0×/week (1.00 ± 0.66). We also observed significantly greater expression of AADAT (kynurenine aminotransferase II) in the 1× ($p = 0.046$), and 3×/week conditions ($p = 0.037$) and a trend toward significance ($p = 0.053$) for the 7×/week group (Fig. 5). No statistically significant differences were observed for any of the target mRNA for the subcutaneous study.

Pre-frontal cortex inflammatory gene expression Statistically significant differences in expression of inflammation-related cytokines were observed in the pre-frontal cortex in the oral study
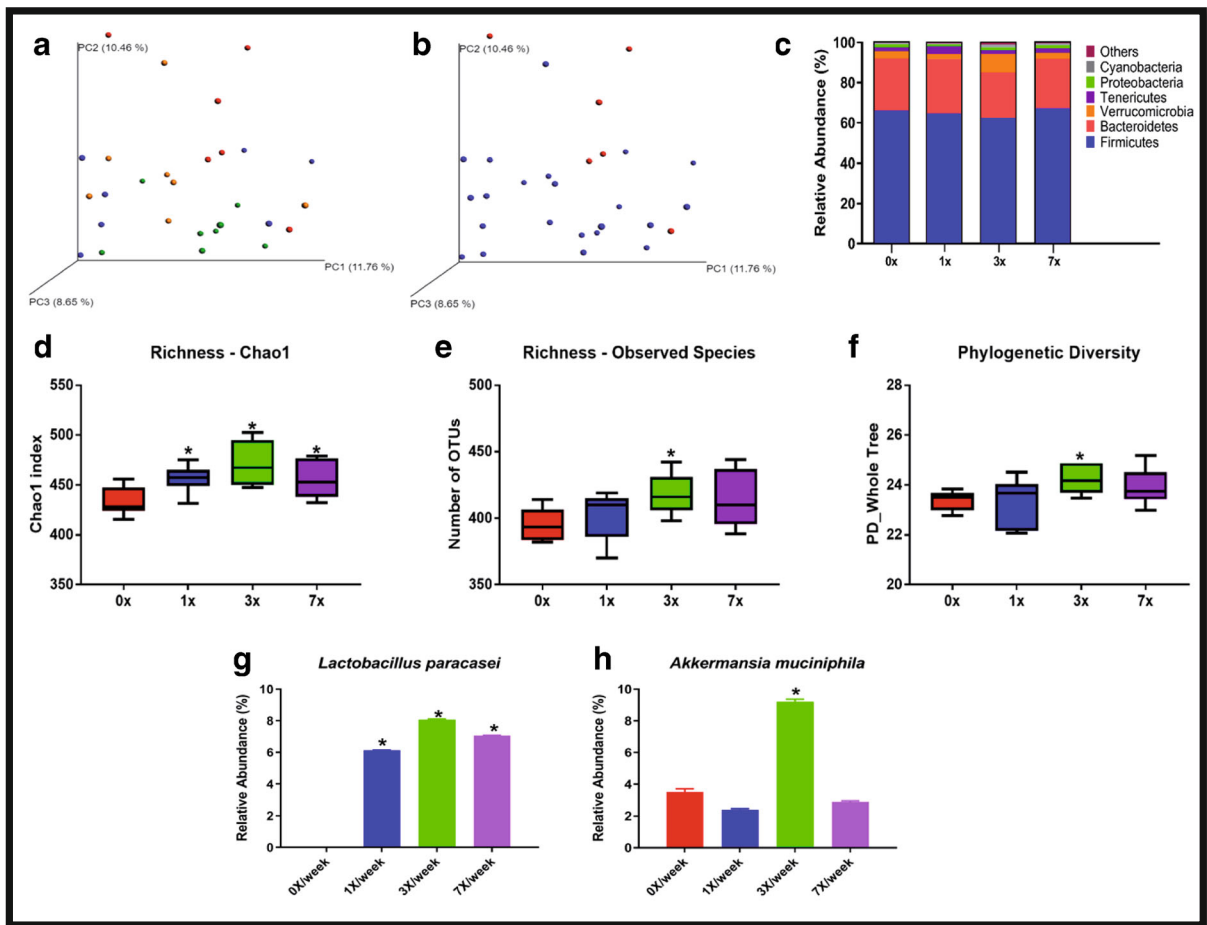
Fig. 3 Key indices of microbiome composition in response to oral LP-A delivery measured at day 29. (A) Comparison of fecal microbiome β-diversity (unweighted UniFrac) by experimental group (0×, 1×, 3×, 7×/week) and (B) by experimental (1×, 3×, 7×/week) vs. control (0×/week). (C) Taxonomic distribution of fecal microbiome of experimental groups at the phylum level. (D, E, F) Comparison of α-diversity of the fecal microbiome among experimental groups including (D) chao1, (E) observed species, and (F) phylogenetic diversity. (G, H) Species-level fecal microbial DNA populations differentially expressed between groups including (G) *Lactobacillus paracasei* and (H) *Akkermansia muciniphila*. Asterisks indicate statistically significant difference from the 0×/week dosing group after correcting for multiple comparisons via false discovery rate

(Fig. 6). The 1×/week regimen significantly reduced COX2 expression ($p = 0.003$), 3×/week reduced COX2 ($p = 0.003$), IL-1β ($p = 0.040$), and TNFα ($p = 0.013$) expression, and 7×/week reduced COX2 ($p = 0.003$) and IL-1β ($p = 0.040$) expression. No statistically significant differences were observed in response to subcutaneous delivery of Ang(1–7) (Supplemental Fig. 4).

Discussion

This manuscript outlined findings from two 4-week dosing studies designed to identify any dose-dependent effects of oral (in form of a genetically modified probiotic) and subcutaneous administration of angiotensin (1–7) and to explore potential differences in indices of gut-brain axis physiology between the two routes of administration. Data from these experiments will be used to inform procedures regarding the optimal delivery method and dose for more comprehensive, longer-term studies in this area. To our knowledge, this is the first study to show potential benefits of a genetically modified probiotic on the gut-brain axis in older animals.

In summary, oral delivery of Ang(1–7) expressing *Lactobacillus paracasei* (LP-A) demonstrated significant alterations in the gut microbiome with concomitant
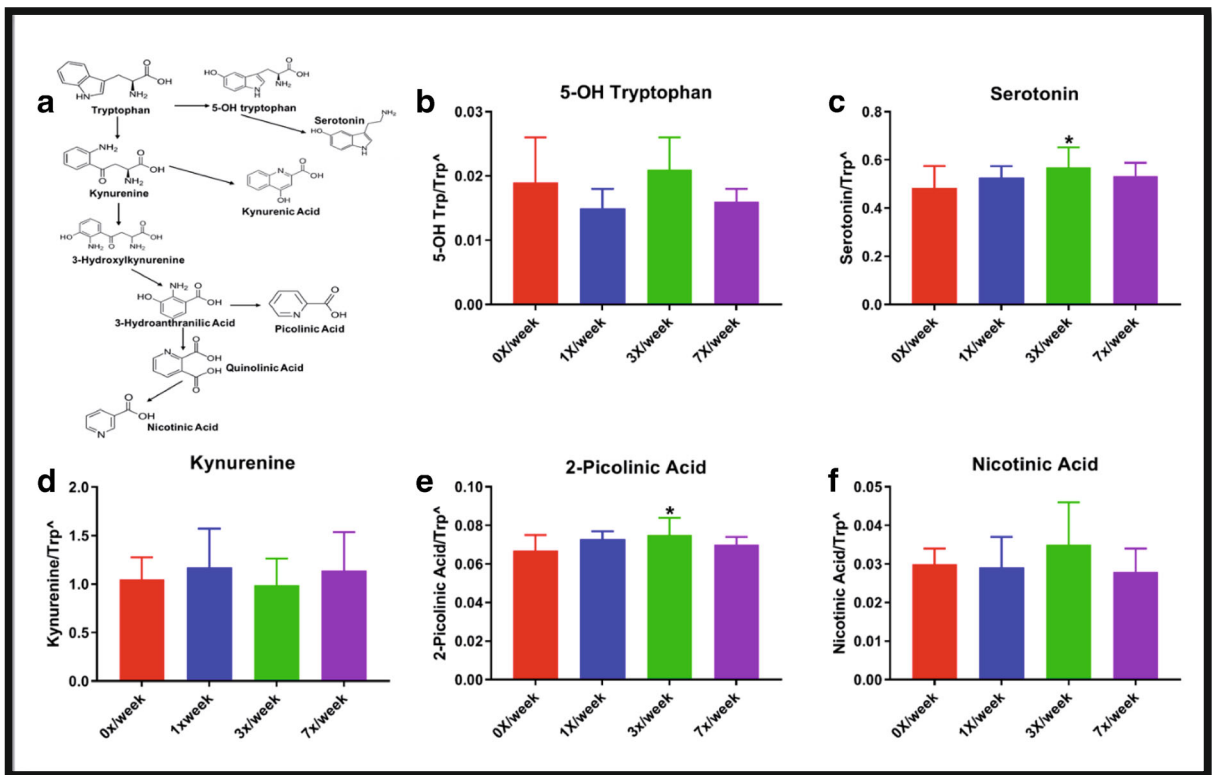
Fig. 4 Serum concentrations of downstream metabolites of tryptophan metabolism genes following 29 days of oral Ang(1–7) expressing *Lactobacillus paracasei* delivery. (A) Simplified schematic of serotonin and kynurenine pathways of tryptophan metabolism and (B) serum 5-OH tryptophan, (C) serotonin, (D) kynurenine, (E) 2-picolinic acid, and (F) nicotinic acid. Carets indicate data expressed as metabolite concentration (mean ± SD) relative to total tryptophan normalized via the square root function (due to non-normally distribution of raw data). Asterisks indicate a significant difference from the 0×/week condition

Fig. 5 Colon mRNA expression of AADAT (kynurenine aminotransferase II) gene following 29 days of oral Ang(1–7) expressing *Lactobacillus paracasei* delivery. Data expressed as relative expression (2^−ΔΔ CT) vs. the 0×/week group (mean ± SEM). *$p < 0.05$; $p = 0.053$ for the 7×/week group
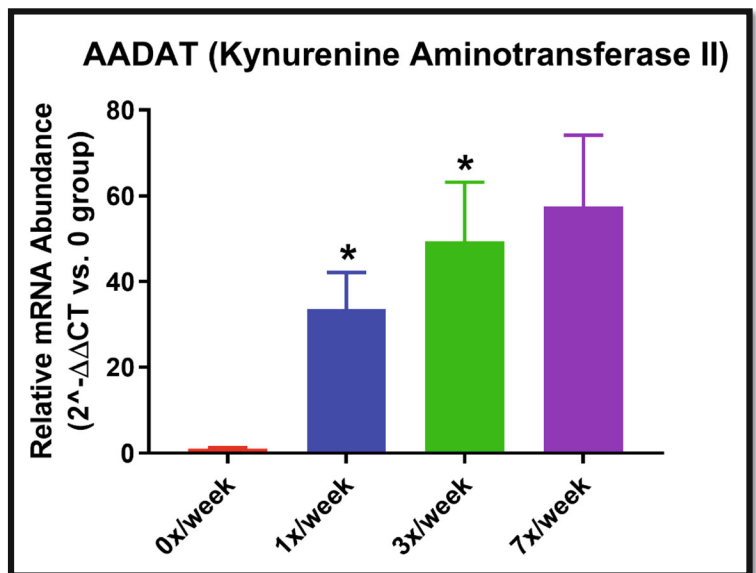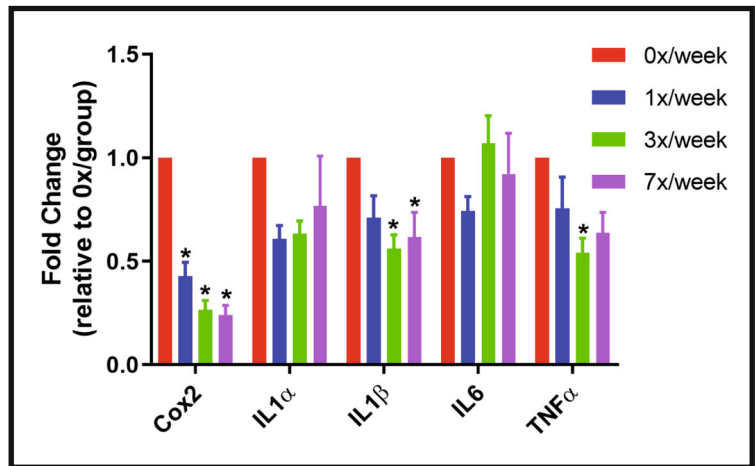
Fig. 6 Pre-frontal cortex mRNA expression of inflammation-related genes following 29 days of oral Ang(1–7) expressing *Lactobacillus paracasei* delivery. Data expressed as relative expression ($2^{-\Delta\Delta\ CT}$) vs. the 0×/week group (mean ± SEM). *$p < 0.05$

increases in beneficial circulating neurotransmitters and decreased neuro-inflammatory gene expression in the pre-frontal cortex. Notably, these changes were most robust in the 3×/week dosing regimen (Fig. 7)—in line with our prior findings on the influence of LP-A on circulating Ang(1–7) (Carter et al. 2019). In contrast, despite significantly increasing circulating Ang(1–7) and related analytes, subcutaneous administration of Ang(1–7) resulted in few changes in gut-brain axis parameters.

A primary premise for the use of LP-A is the concomitant actions of both a probiotic bacteria and Ang(1–7)—each which has independent effects on the central nervous system. Indeed, others have purported the potential benefits and therapeutic applications of genetically modified probiotics (GMPs), particularly those from lactic acid bacteria (LeBlanc et al. 2013;

Bermudez-Humaran et al. 2013; Cano-Garrido et al. 2015; Plavec and Berlec 2019; Borner et al. 2019). Major benefits of this approach to drug delivery include (1) typical inherent benefits of the bacteria itself, (2) ease of production, (3) ability for oral administration due to the ability of the bacteria to survive gut digestive processes, and (4) ability to influence both systemic and mucosal immune responses. Thus, GMPs are considered a major opportunity in biotherapeutic development (Bron and Kleerebezem 2018; Mays and Nair 2018). Interestingly, the 3×/week delivery of LP-A induced significant increases in fecal expression of *Akkermansia muciniphila*, an anti-inflammatory bacterial species known to improve gut barrier integrity and associated with reduced prevalence of several age- and inflammation-related conditions (Everard et al. 2013; Reunanen et al. 2015; Schneeberger et al. 2015; Dao
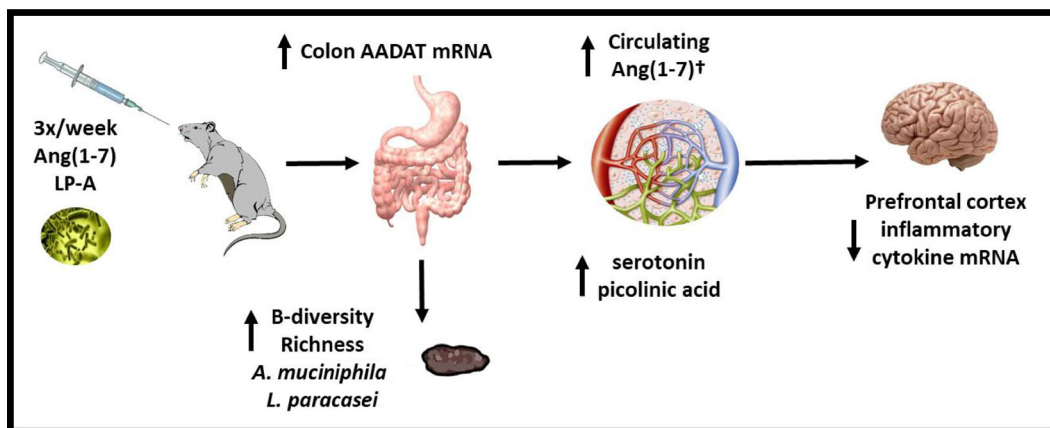

Fig. 7 Summary of changes observed with 3x/week dosing of oral Ang(1–7) expressing *Lactobacillus paracasei* (LP-A) delivery. The dagger indicates changes in circulating Ang(1–7) were published previously (Carter et al. 2019)

et al. 2016; Fransen et al. 2017; Plovier et al. 2017; Hanninen et al. 2018; Chelakkot et al. 2018; Grander et al. 2018; Anhe et al. 2019). Given the consistently beneficial outcomes ascribed to increases in this bacterial species, our finding of an increase through LP-A administration may represent one mechanism involved in the observed decrease in neuro-inflammatory gene expression—though further follow-up is needed to confirm.

Components of the Ang(1–7) axis are found throughout the central nervous system (CNS) including within neurons, astrocytes, cerebral arteries, and various brain regulatory centers (Young et al. 1988; Chappell et al. 1989; Hamming et al. 2004; Gallagher et al. 2006; Doobay et al. 2007; Becker et al. 2007; Guo et al. 2010). Notably, Ang(1–7) stimulates numerous molecular pathways responsible for beneficial actions which could contribute to improved cognitive function including increased (1) endothelial nitric oxide synthase (eNOS), (2) brain-derived neurotrophic factor (BDNF), and (3) vascular endothelial growth factor (VEGF) as via reducing oxidative stress and inflammation (Passos-Silva et al. 2013; Zheng et al. 2014; Jiang et al. 2014; X. L. Wang et al. 2016a; Kamel et al. 2018). Similarly, probiotics are thought to influence the CNS via modulation of the gut microbiota which can communicate with the CNS in several different ways including (1) release of pro-inflammatory cytokines to activate the hypothalamic-pituitary-adrenal (HPA) axis or directly impact CNS immune activity, (2) production of short-chain fatty acids, (3) release of neurotransmitters, or (4) by modulating tryptophan metabolism and downstream metabolites (Sharon et al. 2016; Hampton 2017; Kennedy et al. 2017; Strandwitz 2018; Fulling et al. 2019).

Interestingly, components of the Ang(1–7) axis are also present in significant amounts within the gut including the small intestinal brush border and muscularis mucosa and propria, as well as microvascular endothelium and vascular smooth muscle cells (Hamming et al. 2004). In fact, the highest tissue concentrations of mRNA for ACE2—the enzyme responsible for producing Ang(1–7) endogenously—are found in the terminal ileum, duodenum, and colon (Tipnis et al. 2000; Harmer et al. 2002). ACE2 is also essential to intestinal absorption of tryptophan (Hashimoto et al. 2012; Singer et al. 2012) as well as in regulating intestinal immune function, ecology of the gut microbiota, and attenuating intestinal inflammation suffered in response to epithelial damage (Kowalczuk et al. 2008; Camargo et al. 2009; Singer et al. 2012;

Hashimoto et al. 2012). Thus, both the *Lactobacillus* strain and the Ang(1–7) in our GMP hold promise for altering these neuro-regulatory pathways.

The changes in the systemic neurotransmitters related to tryptophan metabolism are one particularly relevant link between LP-A and the gut-brain axis. The vast majority (~95%) tryptophan is absorbed in the intestines for transport to the liver and release into the circulation. Once released into the circulation, a minority of this tryptophan is broken down to support protein synthesis and the production of serotonin while the vast majority is metabolized to the neuro-regulatory kynurenine pathway. This pathway is proposed as a key link between gut dysbiosis and various neurologic conditions including AD, with elevated kynurenine proposed as a risk factor for neurodegeneration while picolinic acid—which was increased in response to 3×/week LP-A administration—proposed as a neuroprotective agent (Widner et al. 2000; Gulaj et al. 2010; Agudelo et al. 2014; Curto et al. 2015; Parrott et al. 2016; Lovelace et al. 2017). Thus, this pathway may contribute to the reductions in neuro-inflammatory gene expression, though further work remains to confirm this hypothesis.

An interesting finding from these studies is that both oral and subcutaneous delivery methods increased circulating Ang(1–7) concentrations, but only the oral method induced major physiologic changes in the indices of the gut-brain axis evaluated. Notably, this outcome should not be taken to limit the potential utility of subcutaneous administration on overall CNS function or cognition as other evidence indicates that Ang(1–7) axis modulation benefits multiple aspects of cognition in several models of cognitive dysfunction (Xie et al. 2014; Lazaroni et al. 2016; L. Wang et al. 2016b; Kangussu et al. 2017; Hay et al. 2017; Hay et al. 2019). Thus, it is likely that subcutaneous administration had other effects on CNS function which we did not measure here. However, subcutaneous delivery of Ang(1–7) requires burdensome infusions and/or repeated injections as well as the expensive and cumbersome production process for the drug. Moreover, unfavorable pharmacologic properties (i.e., extreme short half-life and rapid clearance in circulation and tissues) may limit its clinical applications. Thus, we were interested in finding a delivery method which could influence CNS function while delivered orally. Thus, the utility of LP-A may be in specifically modulating gut-mediated pathways of CNS function.

One lingering question from this study is whether the impact of LP-A was due solely to the bacteria itself or due to the combination. Thus, subsequent studies are still needed to compare the effects of LP-A with unmodified *Lactobacillus paracasei*. Moreover, given that a primary objective of these studies was to inform regarding dosing and delivery for larger-scale studies, there remains much to learn beyond these studies. For instance, the LP-A compound should be evaluated for a longer time period with behavioral measurements and more comprehensive physiology to more fully identify therapeutic potential. Secondly, studies will need to be performed with both sexes as differences between males and females have been observed in both circulating and tissue levels Ang(1–7) (Pendergrass et al. 2008; Sullivan et al. 2010). Third, evaluation of the impact of potential effect modulators such as diet and exercise may hold critical importance for ultimately translating to humans. Finally, studies in more formal models of Alzheimer's disease or other dementias may also hold meaningful insight for ultimately aiding in therapeutic development.

In summary, administration of LP-A—but not subcutaneous—Ang(1–7) to 24-month-old F344/BN rats altered several physiologic parameters relevant to gut-brain communication. Strongest effects were observed with 3×/week oral dosing; thus, we will utilize this dosing regimen for subsequent studies in this area.

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