Distinct Commensal Bacterial Signature in the Gut Is Associated with Acute and Long-Term Protection from Ischemic Stroke

Distinct Commensal Bacterial Signature in the Gut Is Associated With Acute and Long-Term Protection From Ischemic Stroke

Corinne Benakis, PhD; Carrie Poon, PhD; Diane Lane, PhD; David Brea, PhD; Giulia Sita, PhD; Jamie Moore, BSc; Michelle Murphy, BSc; Gianfranco Racchumi, BSc; Costantino Iadecola, MD; Josef Anrather, VMD

Background and Purpose—Commensal gut bacteria have a profound impact on stroke pathophysiology. Here, we investigated whether modification of the microbiota influences acute and long-term outcome in mice subjected to stroke.

Methods—C57BL/6 male mice received a cocktail of antibiotics or single antibiotic. After 4 weeks, fecal bacterial density of the 16S rRNA gene was quantitated by qPCR, and phylogenetic classification was obtained by 16S rRNA gene sequencing. Infarct volume and hemispheric volume loss were measured 3 days and 5 weeks after middle cerebral artery occlusion, respectively. Neurological deficits were tested by the Tape Test and the open field test.

Results—Mice treated with a cocktail of antibiotics displayed a significant reduction of the infarct volume in the acute phase of stroke. The neuroprotective effect was abolished in mice recolonized with a wild-type microbiota. Single antibiotic treatment with either ampicillin or vancomycin, but not neomycin, was sufficient to reduce the infarct volume and improved motorsensory function 3 days after stroke. This neuroprotective effect was correlated with a specific microbial population rather than the total bacterial density. In particular, random forest analysis trained for the severity of the brain damage revealed that Bacteroidetes S24.7 and the enzymatic pathway for aromatic metabolism discriminate between large versus small infarct size. Additionally, the microbiota signature in the ampicillin-treated mice was associated with a reduced gut inflammation, long-term favorable outcome shown by an amelioration of the stereotypic behavior, and a reduction of brain tissue loss in comparison to control and was predictive of a regulation of short-chain fatty acids and tryptophan pathways.

Conclusions—The findings highlight the importance of the intestinal microbiota in short- and long-term outcomes of ischemic stroke and raises the possibility that targeted modification of the microbiome associated with specific microbial enzymatic pathways may provide a preventive strategy in patients at high risk for stroke.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2020;51:1844-1854. DOI: 10.1161/STROKEAHA.120.029262.)**

Key Words: ampicillin ■ bacteroidetes ■ metabolic pathways ■ microbiota ■ stroke ■ vancomycin

Commensal bacteria that populate epithelial surfaces play a defining role in the physiology, metabolism, and immunity of the host, depending on the relative abundance, the composition, and function of microbial species.[1,2] Environmental factors including the type of diet, exercise, use of medications as well as nonmodifiable factors such as the host genetics, mode of birth delivery, and age influence the gut microbiota composition. In turn, changes in the microbiome may impact the host homeostasis and influence health and diseases.[1,3] Advances in metagenome sequencing of the microbiome have allowed the identification of noncultivable bacteria and have revealed a close association of this complex ecosystem with multiple diseases.[4] Whereas human research has mostly addressed the correlative link between the microbiome and disease state, the development of animal models and fecal microbiota transplantation have provided evidence as to showing a direct bidirectional communication between the microbiome and the brain.[5] In particular, recent studies have implied that the microbiome can have remarkable effects on brain diseases, including Alzheimer disease,[6] Parkinson disease,[7] multiple sclerosis,[8] neurodevelopmental[9] and psychiatric disorders[10,11] as well as stroke.[12–18]

Signaling between the brain and the gut occurs through neuronal pathways but also through microbial metabolites as

Received October 10, 2019; final revision received March 19, 2020; accepted April 13, 2020.

From the Feil Family Brain and Mind Research Institute, Weill Cornell Medical College, New York.

Guest Editor for this article was Miguel A. Perez-Pinzon, PhD.

The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/STROKEAHA.120.029262.

Correspondence to Corinne Benakis, PhD, Institute for Stroke and Dementia Research (ISD), Klinikum der Universität München, Feodor-Lynen-Straße 17, Munich 81377, Germany, Email corinne.benakis@med.uni-muenchen.de or Josef Anrather, Feil Family Brain and Mind Research Institute Weill Cornell Medicine, 407 E 61st St RR409, New York, NY 10065, Email joa2006@med.cornell.edu

well as hormones, and the immune system.[19] Importantly, the vast majority of the microorganisms reside in the gastrointestinal tract and tightly regulate immune cell function.[20] We and others have shown that the gut microbiome influences stroke outcome by modulating the immune response,[16] in turn stroke itself induces a shift of the microbial community which impacts gut motility and permeability, stress response, and poststroke infection.[13,17,18,21] In particular, these findings highlight a direct connection along the gut-brain axis via intestinal T cells regulating the neuroinflammatory response to brain injury.[22]

The bacterial species underlying the protective or deleterious effect in stroke remain to be defined, as well as whether a shift of the microbiota composition has a long-lasting impact on stroke outcome. Recent findings from Spychala et al[23] have identified that the microbiome from aged mice is associated with a disbalance of the 2 main bacteria phyla in the gut in comparison to young mice and fecal transplantation of a young microbiome in aged mice improved stroke outcome, suggesting that targeting the microbiome composition in patients with high risk of stroke, such as in elderly might be beneficial. Understanding on how modification of the gut microbial community impacts the consequences of stroke on the host has the potential for the development of new therapeutic strategy to improve recovery after stroke.

In this study, we use combinatorial and singular antibiotic treatment protocols to modify the microbiota composition. We showed that a targeted modification of the gut microbiota is associated with acute and long-term protection from ischemic stroke.

Methods

Raw sequence reads were deposited to the NIH Sequence Read Archive (SRA) with the accession No. PRJNA613289. All other data that support the findings of this study are available from the corresponding authors upon reasonable request.

Detailed experimental description of the different materials and methods described below can be found in the Data Supplement.

Mice and Antibiotic Treatment

All procedures were approved by the institutional animal care and use committee of Weill Cornell Medical College (WCMC, protocol number: 2012-0051). Wild-type C57BL/6 4 weeks old male mice were purchased from Jackson Laboratories (JAX; Bar Harbor, ME) and housed under standard conditions of our WCMC animal facility. Antibiotic treatment: ampicillin, metronidazole, neomycin sulfate, and vancomycin, abbreviated AMNV, was administered for 4 weeks in the drinking water. Because of the possible effect of sex hormones on the microbiota composition, experiments were performed in male mice. All animal experiments were performed in accordance with the animal research: reporting of in vivo experiments guidelines.[24]

Middle Cerebral Artery Occlusion and N-methyl-D-Aspartate Lesion

Transient middle cerebral artery occlusion (MCAO) was induced as previously described with monitoring of body temperature (37.0±0.5) and by transcranial laser Doppler flowmetry (Tables in the Data Supplement).[16] Topical lidocaine/bupivacaine (0.25 %/0.1 mL, transdermal) and buprenorphine (0.5 mg/kg; subcutaneously) were used for pre- or postoperative analgesia, respectively. *N*-methyl-D-aspartate (NMDA) lesion was induced into the parietal cortex as described previously.[25] The order in which mice from different groups were subjected to surgery was randomized. Antibiotic treated mice cannot be blinded to the investigator because they have an enlarged abdomen, and their stool pellets seem distinguishable from control mice.

Quantification of Lesion Volume and Tissue Loss

Mice were euthanized 3 days or 5 weeks after MCAO for infarct volume and tissue loss quantification, respectively, or 1 day after NMDA injection for lesion volume measurement.

Neurobehavioral Testing

Sensorimotor deficit was assessed 1 day before and 3 days after MCAO using the Tape Test.[16] The open field test was used to assay spontaneous locomotor activity 14 days after MCAO or sham surgery.

Feces DNA Extraction, 16S rRNA Gene Amplification, and Quantification

DNA was extracted from frozen stool samples, amplified using 16S-V2 primers and quantitated by real-time polymerase chain reaction.

16S rRNA Gene Amplification and Multiparallel Sequencing, Analysis and Visualization

Amplicons of the V4 16S rRNA region were amplified and sequenced using an Illumina MiSeq platform (LC Sciences, Houston, TX). Demultiplexed sequence reads and metabolic pathways from the taxonomic composition were processed with the QIIME2 (vers. 2019.4) pipeline[26] and implemented in Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt).[27]

Statistical Analysis

Mouse randomization was based on the random number generator function (RANDBETWEEN) in Microsoft Excel software. Exclusion criteria are described in the individual method sections. GraphPad Prism (v. 8.0) software was used for statistical analysis. Data are expressed as mean±SD or SEM and were analyzed by unpaired Student *t*-test (2-tailed), or 1-way ANOVA and Tukey test, as appropriate. Significant differences are indicated by: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ or nonsignificant: NS; details of the mean and N values/group can be found in the Data Supplement.

Results

Antibiotic Treatment Induces Neuroprotection and Is Reversible by Passive Recolonization

A cocktail of antibiotics targeting a broad spectrum of gut bacteria was administered to mice to evaluate its effect on fecal bacterial density and stroke outcome. Mice received AMNV in the drinking water for 4 weeks. AMNV treatment was discontinued 3 days before inducing MCAO to mitigate off-target antibiotic effects. Control C57BL/6J mice (CTR) were age matched and received autoclaved water. Stool pellets were collected after 4 weeks of AMNV treatment, at the time of MCAO induction and 3 days after stroke (timeline of the treatment protocol is shown in Figure 1A). As previously reported,[28] we observed that mice treated with AMNV significantly lost weight during the first 3 weeks (death rate of 2.7%) and regained their body weight similar to CTR mice after the 4th week of antibiotics (no mortality was observed at this time point). At the time we induced stroke, there was no significant difference in body weight between the 2 groups (Figure 1B). Quantification of 16S rRNA copies in feces revealed a 10^5-fold reduction in bacterial density 4 weeks after AMNV compared with CTR mice. Bacterial density of AMNV-treated mice was

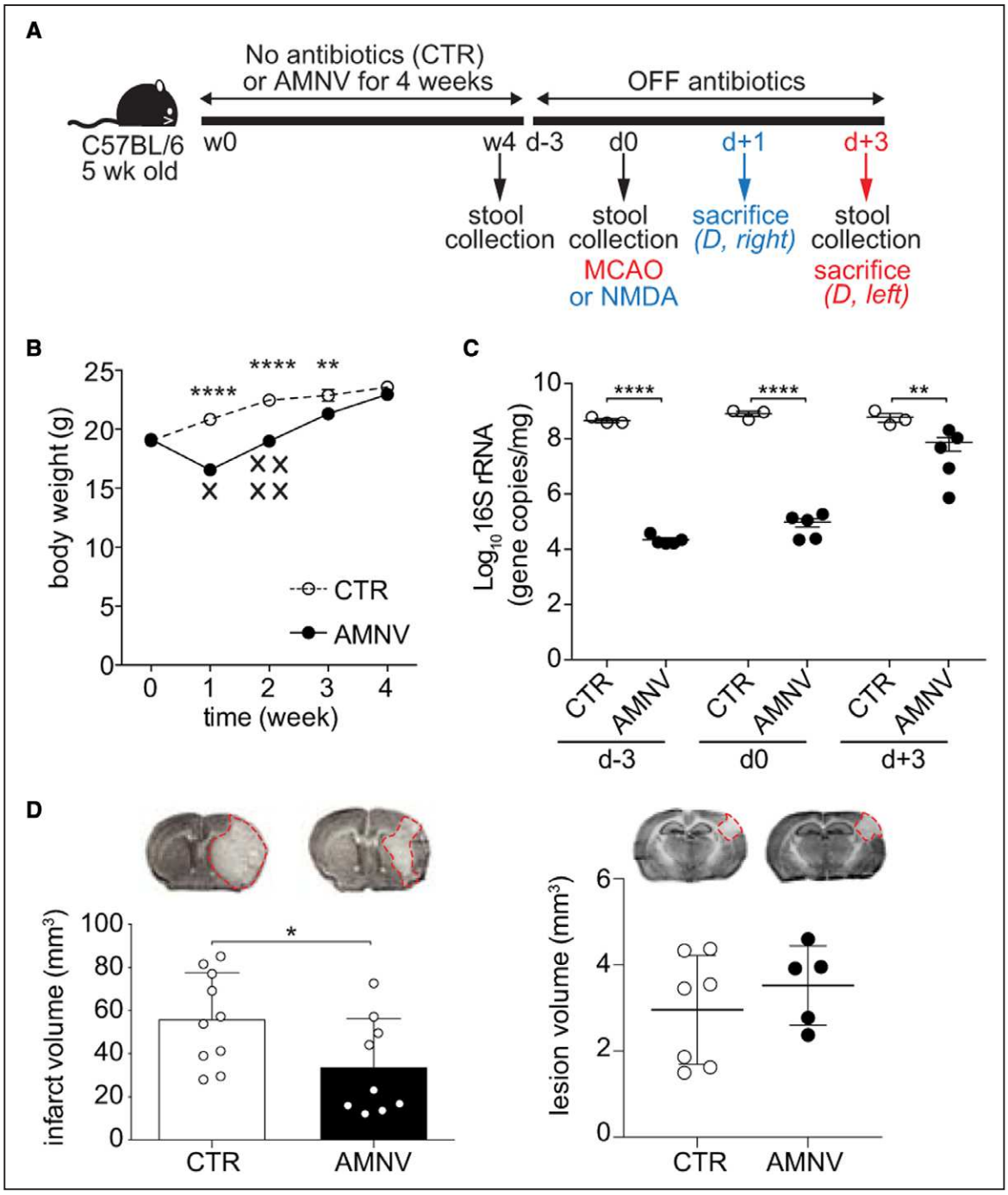

Figure 1. Broad-spectrum antibiotics modifies the bacterial density in feces and induces neuroprotection after stroke. **A**, Mice received broad-spectrum antibiotics ampicillin, metronidazole, neomycin sulfate, and vancomycin (AMNV) in the drinking water for 4 wks (w4). AMNV treatment was discontinued 3 d before inducing middle cerebral artery occlusion (MCAO) or *N*-methyl-D-aspartate (NMDA) injection. Control mice (CTR) were age matched and received autoclaved water. Mice were sacrificed 3 d after MCAO induction or 1 d after NMDA injection. Stool pellets were collected as indicated. **B**, Body weight progression during AMNV treatment. X indicates mice which died during the AMNV treatment. **C**, Fecal bacterial density at several time points after the antibiotic supplementation was discontinued. **D**, Left, infarct volumetry in CTR and AMNV mice 3 d after MCAO induction. Right, lesion volumetry in CTR and AMNV mice 1 d after neocortical injection of NMDA.

largely restored to a 10-fold difference from CTR mice 6 days after antibiotic treatment was stopped (Figure 1C). AMNV treatment for 4 weeks induced a 40% reduction of the infarct volume compared with CTR mice 3 days after MCAO induction (Figure 1D). This shows that combinatorial antibiotics before inducing stroke successfully reduced the bacterial density in feces and was sufficient to induce neuroprotection. To investigate whether changes in microbiota directly influence neuronal death, we used a cortical excitotoxic lesion model.[25] We found that AMNV-treatment had no effect on the size of the brain lesion produced by neocortical NMDA injection. This finding could suggest that the reduction of ischemic injury is not primarily mediated by the inhibition of excitotoxicity (Figure 1D) but rather by modulation of the postischemic

immune response as showed here (Figure IB and IC in the Data Supplement) and published before.[13,16]

We then addressed whether acute antibiotic treatment is neuroprotective in comparison with long-term AMNV treatment. AMNV was given daily to naïve mice starting 1 day prior MCAO until sacrifice at day 3 or to mice treated with AMNV for 4 weeks (Figure 2A). Fecal bacterial density was successfully reduced after short time AMNV treatment similarly to AMNV chronic treatment level (Figure 2B). However, short-time AMNV treatment did not induce a reduction of the infarct volume compared with long-time AMNV treatment (Figure 2B). To investigate the direct link between the gut microbiota and stroke outcome, we passively recolonized AMNV-treated mice by co-housing with a wild-type mouse for 2 weeks (Figure 2C). Fecal bacterial density of the recolonized AMNV-treated mice was re-established to levels found in untreated wild-type mice (1×10^9 copies/mg), and the observed neuroprotection was abolished (Figure 2D), showing the neuroprotective effect is reversible by passive recolonization of the gut microbiota.

Single Antibiotic Treatment Induces a Distinct Bacterial Signature Associated With Improvement of Acute and Long-Term Stroke Outcome

Each antibiotic used in this study has different antimicrobial properties. Here, we wanted to better characterize the effect of singular antibiotic treatment to identify the most potent strategy to alter the composition of the gut microbiota associated with an improvement of stroke outcome. Mice were treated for 4 weeks with either neomycin (N), vancomycin (V), or ampicillin (A), and feces were collected for 16S rRNA sequencing (Figure 3). The antibiotic-treated mice had an overall reduced Shannon diversity index, which its magnitude was dependent on the type of antibiotic (Figure 3A). The reduction of the richness and evenness was more pronounced in V- and A-treated mice (Figure 3B). Specifically, the evenness index in A-treated mice was the lowest in comparison to all groups indicating the dominance of one or few species among the other (Figure 3B). Taxonomic relative abundance of bacterial phyla revealed a contraction of members of the Bacteroidetes in both V- and A- treated mice, as well as an expansion of the antimicrobial-resistant *Verrucomicrobia* and *Proteobacteria*, respectively. Neomycin reduced slightly the diversity but did not influence the overall bacterial population in the stool in comparison to CTR mice (Figure 3A through 3C and Figure IA in the Data Supplement). Overall, redundancy analysis ordination at the family level showed distinct clusters, distinguishing clearly the 3 different antibiotic treatment groups and controls (Figure 3D).

The neuroprotection in AMNV-treated mice was not because of additive effects of the different antibiotics (Figure 4A) because treatment with V or A alone reduced infarct volume 3 days after MCAO induction, whereas N-treated mice were not protected in comparison to CTR mice (Figure 4B). Metronidazole (M) in combination with N or V did not further influence the size of the lesion (data not shown). Body weight progression was similar in all groups tested, and no mortality was recorded during the 4-week treatment protocol. Bacterial load was moderately reduced in V- and A-treated mice (10^2-fold) in comparison to control; however, the neuroprotective effect in V- and A-treated mice were not correlated with the total fecal bacterial density (Figure 4C) but was associated with distinct bacteria dependent on the antibiotic treatment used (Figure 3D and Figure II in the Data Supplement). Reduction of Bacteroidetes S24-7 in both V- and A-treated mice and expansion of *Verrucomicrobiaceae* and *Lactobacillaceae* or *Burkholderiaceae* and *Streptococcaceae* families in V- and A-treated mice, respectively, was correlated with a reduction of brain infarct (Figure 3C). We then tested whether the observed changes of the microbiota composition influenced neurological deficits in the acute phase of stroke. Sensorimotor function was tested 3 days after MCAO by the Tape Test (Figure 4D). Mice treated with either V or A took less time on average to sense and remove the tape on their contralateral forepaw than N-treated and CTR mice in respect to the performance before MCAO induction. Altogether, whereas single antibiotic treatment induced a distinct shift of the microbiota population, V and A treatment but not N were associated with a better stroke outcome (Figure 4) and correlated with a suppression of IL17+ γδT cells in the gut (Figure IB and IC in the Data Supplement).

We then investigated whether the neuroprotective phenotype in mice treated with single antibiotic persisted in the chronic phase of stroke (Figure 5A). Long-term deficits were tested 14 days after stroke by measuring the mobility and exploratory behavior (Figure 5B). Whereas, the total ambulatory distance was not different between groups (Figure IIA in the Data Supplement), A-treated mice tended to develop decreased stereotypic behaviors (time and counts) in comparison to all other groups as shown by a less repetitive behavior in the open field in the presence of an intruder mouse. This decrease reflects a diminution of circling behavior or repetitive back and forth motion as well as a decrease of social stress response (number of entries in the intruder zone and duration in the intruder zone, Figure IIB and IIC in the Data Supplement). In contrary, N-treated mice had an increase of the time spending in the intruder zone in the open field in comparison to CTR mice, suggesting an impaired social behavior in N-treated mice (Figure IIC in the Data Supplement). In addition, the brain tissue loss was less pronounced in A-treated mice 5 weeks after stroke induction as shown by a significant decrease of the volume of the ipsilateral hemisphere in comparison to CTR mice (Figure 5C).

Enzymatic Pathways Associated With a Specific Microbiota Are Predictive of Stroke Outcome

We then used PICRUSt[27] to infer the functional composition of the collective metagenome for each treatment group from the taxonomic data. To identify a predictive microbial signature for stroke outcome, we performed random forest analysis on fecal OTUs at the family level (Figure 6A) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enzymatic pathways (Figure 6B) trained for the severity of the brain damage. We found that the *Bacteroidetes S24.7* and the enzymatic pathway related to xenobiotic metabolism/aromatic biodegradation discriminate highly between large versus small infarct

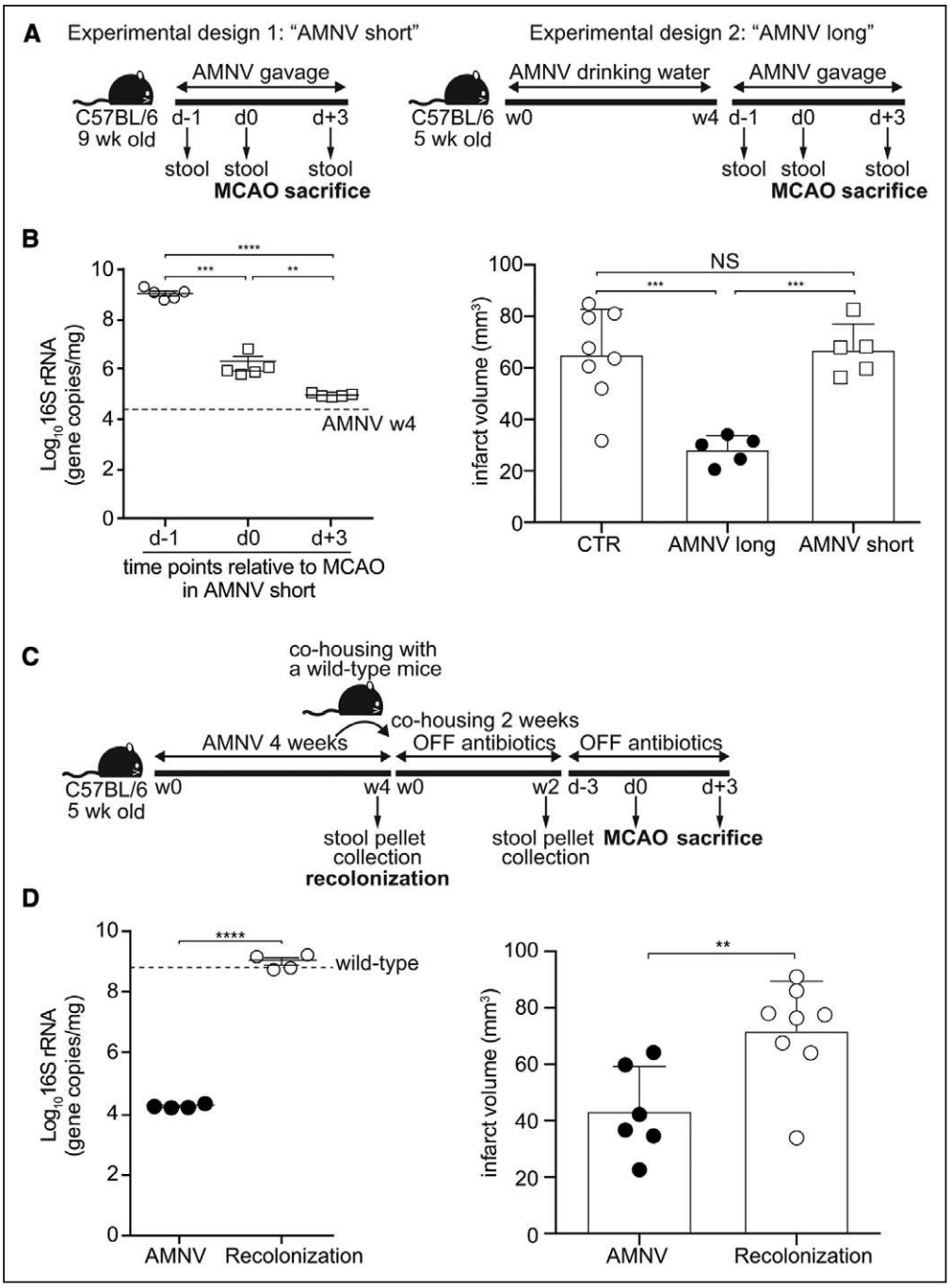

Figure 2. Acute ampicillin, metronidazole, neomycin sulfate, and vancomycin (AMNV)-treatment is not protective from stroke and passive recolonization abolishes neuroprotection. **A**, Left, mice received AMNV by gavage starting 1 d before middle cerebral artery occlusion (MCAO) surgery (d-1) until sacrifice (d+3) (AMNV short). Right, another group of mice received AMNV in the drinking water for 4 wks following by the same treatment procedure as in the left (AMNV long). **B**, Left, bacterial density in feces before and 1 to 3 d after AMNV treatment by gavage (AMNV short). Dash line indicates bacterial density in mice treated with AMNV in the drinking water for 4 wks. Right, infarct volume in control mice not treated (CTR), after chronic AMNV treatment (AMNV long) and acute AMNV treatment (AMNV short) 3 d after MCAO induction. **C**, Four week AMNV-treated mice were recolonized by co-housing for 2 wks with a control wild-type mouse. Stool pellets were collected before and after recolonization. MCAO was induced after 2 wk of co-housing. Mice were sacrificed 3 d after stroke and infarct volumetry was assessed. **D**, Left, bacterial density in feces 4 wks after AMNV administration (black circles) and after 2 wks of co-housing with a wild-type mouse (white circles). Dash line indicates bacterial density in wild-type mice. Right, infarct volume 3 d after MCAO in 4 wk AMNV-treated mice and after recolonization.

size, independently of the antibiotic treatment (Figure 6A and 6B). We then performed a clustered heatmap of KEGG analysis to investigate the biological pathways that were predictive of the different microbiomes. V- and A-treated groups clustered away from the CTR group, whereas N-treated mice were mostly mixed with CTR mice (Figure 6C). Although V-treated mice had only 3 pathways significantly different from the CTR group and N-treated group had none, A-treated mice, which showed the highest and reproducible level of neuroprotection in the acute and chronic phase of stroke, had

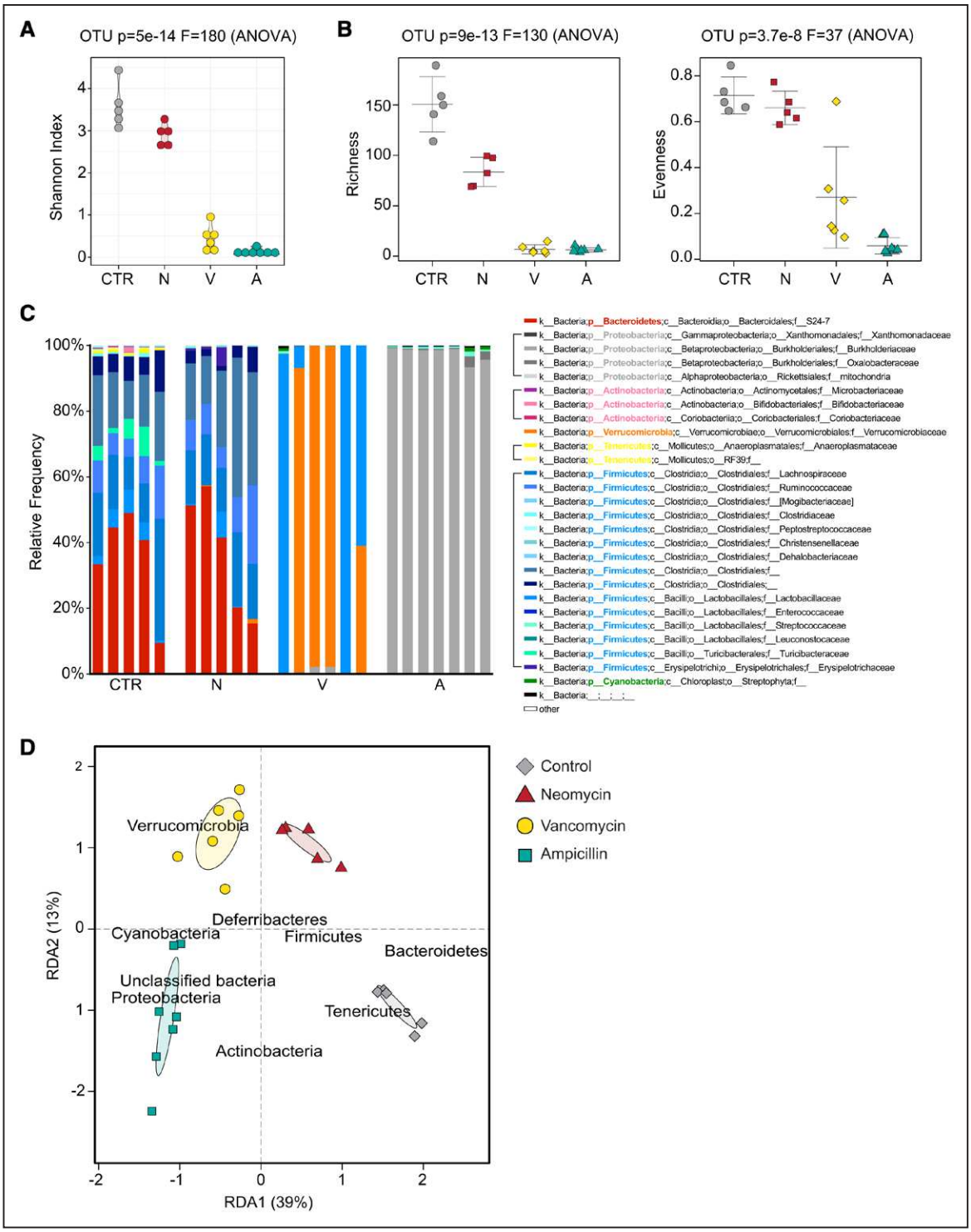

Figure 3. Single antibiotic treatment induces a distinct microbiota signature. **A**, Shannon α-diversity index and (**B**) richness and evenness of OTUs in control mice (CTR) and in mice treated with either neomcyin (N), vancomycin (V), or ampicillin (A) for 4 wks. **C**, Relative abundances of bacterial families in fecal samples 4 wks after the indicated antibiotic treatment. Each bar represents an individual mouse. **D**, Redundancy analysis ordination of the family composition across the 3 antibiotic treatment groups and controls. RDA1 and 2 explained 39% and 13% of the variance, respectively. For better clarity, phyla are indicated on the plot. Defined bacteria phyla are associated with specific antibiotic treatment groups and are distinct from the control group.

40 enzymatic pathways significantly regulated in comparison to CTR mice (Figure III in the Data Supplement). These included an increase of pathways associated with amino-acid metabolism (valine, leucine, isoleucine degradation) and especially the aromatic amino-acids (phenylalanine metabolism, tryptophan metabolism, histidine metabolism), aromatic compound degradation (benzoate degradation), ATP production (glyoxylate and dicarboxylate metabolism, fatty acid degradation, oxidative phosphorylation), carbohydrate metabolism (butanoate metabolism, propanoate metabolism), pyruvate

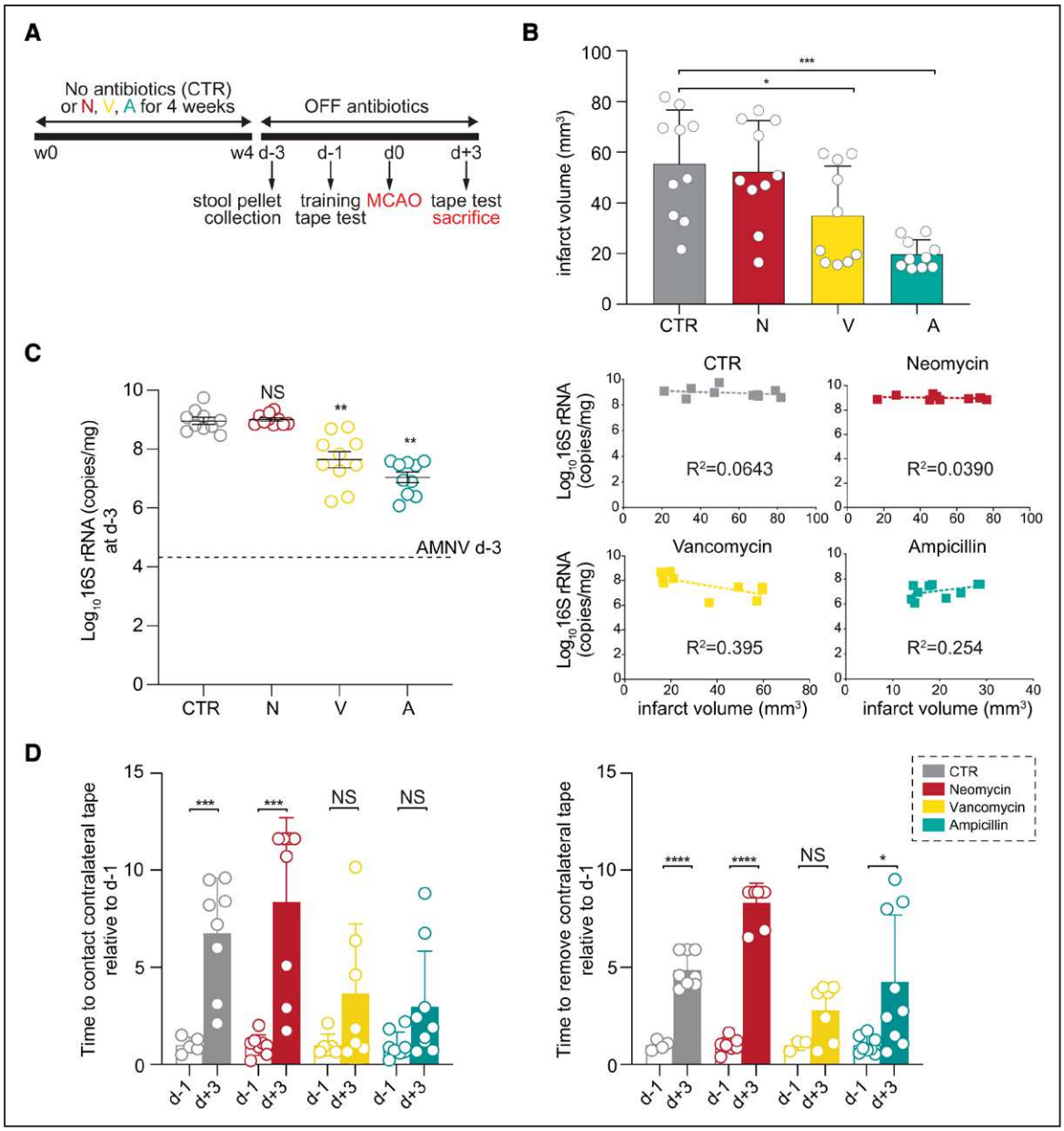

Figure 4. Vancomycin or ampicillin alone is sufficient to improve acute stroke outcome. **A**, Timeline of the single antibiotic administration. One day prior stroke mice were trained for the Tape Test. Three days after stroke, mice were tested for motor and sensory function by the Tape Test. **B**, Infarct volumes in antibiotic treated mice as compared with control (CTR) mice 3 d after middle cerebral artery occlusion (MCAO) induction. **C**, Left, bacterial density in the feces 4 wks after single antibiotic supplementation or in CTR mice. Dash line indicates bacterial density in mice treated with ampicillin, metronidazole, neomycin sulfate, and vancomycin (AMNV) in the drinking water for 4 wks. Right, correlation analysis of the fecal bacterial density with infarct volumes 3 d post-MCAO after 4 wks of single antibiotic treatment or in control mice. **D**, Sensorimotor function in CTR and antibiotic treated mice. Graphs show contact time (left) and time to remove the tape (right) from the contralateral forepaw 3 d after MCAO induction as relative to d-1.

and nitrogen metabolisms. These findings show that modification of the composition of the gut microbiota with ampicillin induces changes of metabolic pathways that could be predictive of stroke outcome.

Discussion

In this study, we used combinatorial and singular antibiotic treatment protocols to modify the composition of the microbiota, and we investigated the acute and long-term effect of gut dysbiosis on stroke outcome. Whereas broad-spectrum antibiotic treatment for 4 weeks prior inducing stroke was neuroprotective, short-time AMNV treatment was inefficient in reducing the size of the ischemic lesion corroborating studies in stroke patients.[29] Additionally, we demonstrated that antibiotics—targeting different classes of intestinal bacteria and metabolic pathways—alter differently the acute and long-term outcome of stroke. Ampicillin-derived microbiome was the most efficient in inducing short- and long-term neuroprotection from stroke and its composition clearly segregated from the other antibiotic-treated mice with no or less efficient protection from stroke. The ampicillin neuroprotective microbiome was associated with an expansion of *Proteobacteria*

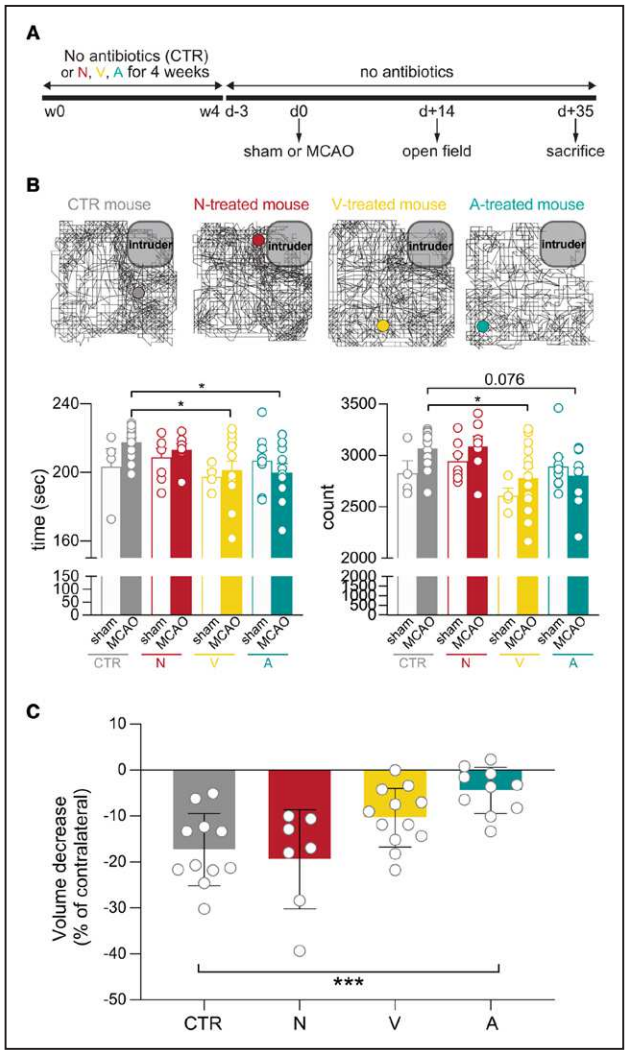

Figure 5. Single antibiotic treatment with vancomycin or ampicillin improves long-term stroke outcome. **A**, CTR and 4-wk antibiotic-treated mice were tested for stereotypic behavior in the vicinity of a foreign mouse (intruder) by the open field test 14 d after stroke or sham surgery. Mice were sacrificed 35 d after middle cerebral artery occlusion (MCAO) induction for quantification of the brain tissue loss. **B**, Upper row shows a representative trajectory of mouse movements during the presence of the intruder mouse at the location indicated. Colored circle indicates the position of the mouse at the end of the 15 min trial. Lower row, quantification of the stereotypic behavior (time and counts) in the open field of CTR and antibiotic-treated mice in presence of the intruder 14 d after stroke and sham surgery. No statistical difference was observed between sham groups. **C**, Brain tissue loss was less pronounced in A-treated mice 35 d after MCAO as reflected by a significant decrease of ipsilateral volume loss compared with CTR mice.

and *Lactobacilliales* and was predictive of specific metabolic pathways but was independent of fecal bacterial density. Thus, bacterial density does not necessarily correlate with stroke outcome. Indeed, short-term antibiotics lack to provide neuroprotection compared with the protection with long-term antibiotics despite a change in the microbiome density in both antibiotic treatment paradigm. We highlighted here that different class of antibiotics induce distinct changes of the microbiota composition and was associated with a distinct immune response in the gut. In particular, antibiotics such as ampicillin and vancomycin but not neomycin promote an anti-inflammatory milieu in the gut by reducing the number of IL-17-producing γδT cells, which was associated with short- and long-term neuroprotection. We have previously shown that the alteration of the microbial composition precedes the immune changes in the intestine.[16] Whether an expansion of a specific bacterial species or family is responsible for this time lag should be addressed in future studies. As for instance, we observed that the bacteria family *S24-7* within the phylum Bacteroidetes was highly associated with the size of the infarct. Different studies have reported the altered abundance of *S24-7* family in association with brain diseases.[30–32] *S24-7*, among other genera, possess large numbers of genes involved in carbohydrate metabolism[33] and have been involved in humoral response[34] in the gut that could impact the availability of gut metabolites for the host and its immune response, respectively. Despite these findings, the specific role of *S24-7* members on stroke outcome has not been tested yet.

In our previous study, we were able to inhibit the development of the infarct when fecal microbiota transplantation of the modified protective microbiota was inoculated to naive mice.[16] However, we could not determine whether the protective phenotype after fecal microbiota transplantation was solely due to the original microorganisms, as the phylogenetic analysis of the transplanted recipient mice was highly different from the donor mice (Benakis et al). This is not surprising, considering that (1) bacteria might not survive the oral gavage and passage through the acidic and aerobe conditions of the upper gastrointestinal tract, (2) colonization of the gut may need empty niches to be efficient, (3) the recipient immune system may not be primed to accept the transplant. However, one can assume that recapitulation of the donor microbiome in the recipient might not be necessary since different bacterial species have redundant functions. Thus, investigation of the metabolic microbial pathways might be a better approach to describe the altered microbiome after fecal microbiota transplantation. Here, using PICRUSt tool as a discovery-based algorithm, we highlighted that the enzymatic pathway related to xenobiotic/aromatic compound degradation discriminate highly between large versus small infarct size. Stanley et al[21] analyzed the predicted KEGG pathways from samples isolated directly from the intestinal mucosal microbiota. In agreement with our study, they found an increasing capacity for xenobiotic biodegradation and metabolism after stroke in comparison to sham surgery. This suggest that xenobiotic/aromatic-degrading microorganisms might be regulated by stroke and involved in the development of the infarct. Interestingly, several pathways related to aromatic compound metabolism (benzoate degradation, phenylalanine, tryptophan and histidine metabolism) were associated with the microbiome composition of the ampicillin-treated mice, which induces the most potent neuroprotective effect after stroke, in comparison to control mice. Although it is not clear yet how regulation of these enzymatic pathways, predicted by PICRUSt algorithm, impact the host and stroke outcome, there is evidence from the literature showing that catabolites of the essential amino acid tryptophan that include indole metabolites can modulate intestinal immune cell function through the aryl hydrocarbon receptor (AHR; first discovered as a receptor for

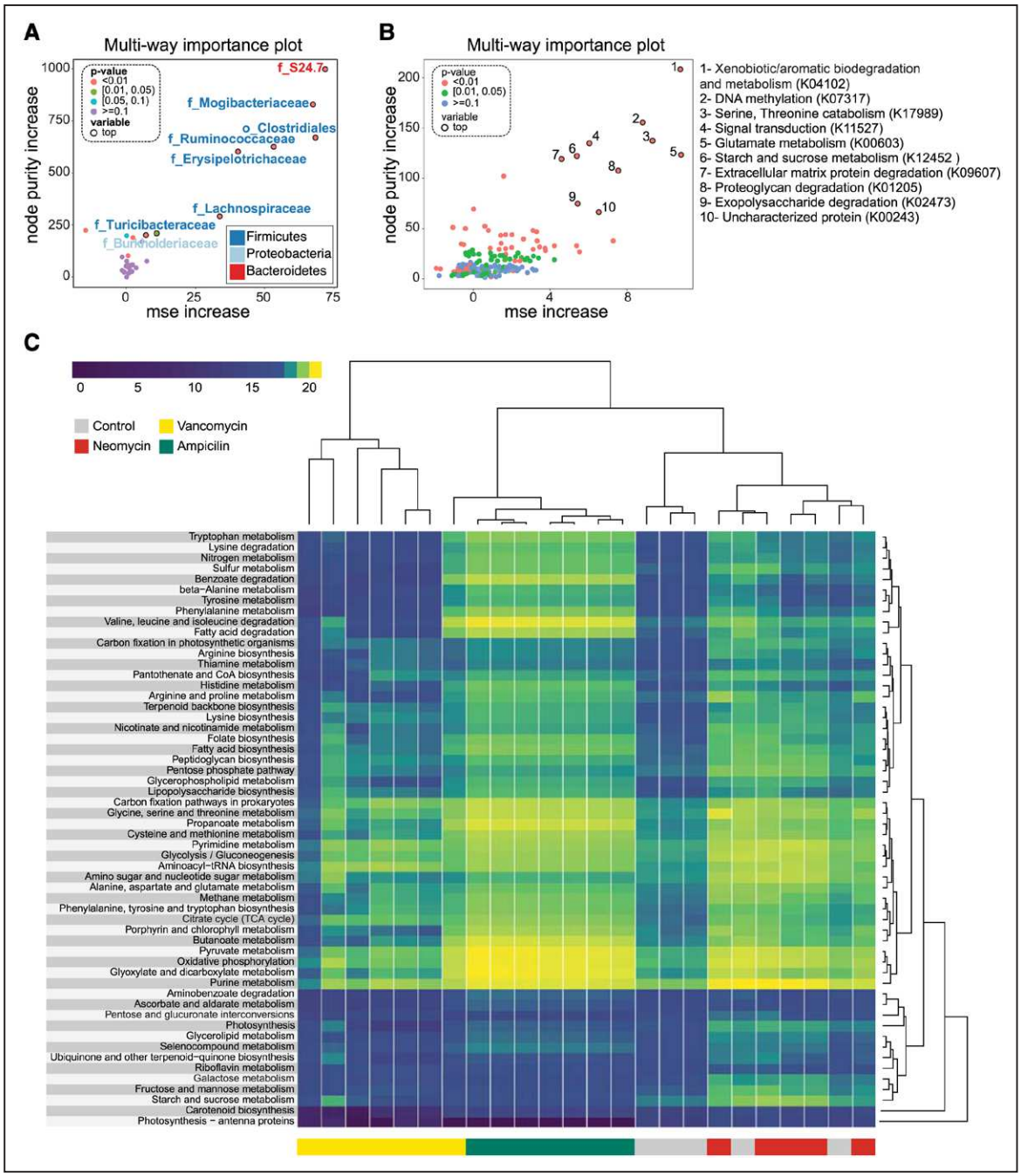

Figure 6. Association of specific bacteria and enzymatic pathways with infarct volume. Random forest (RF) analysis showing predictive importance of (**A**) operational taxonomic units (OTU) at the family level (OTUs >1% were included) and (**B**) enzymatic Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways trained for stroke outcome (size of the infarct volume) in all samples from the antibiotic treated- and control groups. mse (mean squared error) is a measure of the prediction accuracy of the RF as a function of permutating a variable. Variables associated with higher mse increase have higher predictive value. Node purity increase is a measure of how a split on a given variable reduces node impurity if applied to all nodes and all trees. Higher values are associated with more important variables. **C**, Heat-map of the significant regulated enzymatic pathways (rows) by samples (columns) after hierarchical clustering of treatment groups and KEGG pathways. Each column represents one mouse.

xenobiotic ligands).[35] In an animal model of multiple sclerosis, Rothhammer et al[8] showed that the brain inflammatory response to the disease was regulated by AHR through indole metabolites.[36] Thus, direct modulation of these pathways by microbial metabolite supplementation or inhibition might have an impact on outcome of stroke. Short-chain-fatty acids are other group of microbial metabolites exclusively produced by bacterial fermentation of dietary fibers. Interestingly, we showed that alteration of the gut microbiota by ampicillin was also found to change the predicted functionality of short-chain-fatty acid metabolism, with a significant increase in pathways of butyrate and propionate metabolisms. In accordance, we have recently demonstrated that short-chain-fatty acids added in the drinking water of mice for 4 weeks influenced microglia morphology toward a resting phenotype, increased neuronal spine growth and was associated with long-term recovery

after stroke,[37] suggesting an important role of gut-derived metabolites in modulating the outcome of stroke.[23,37]

The microbiome is a highly complex ecosystem with dynamic interactions between the different types of bacteria. Investigating the microbiome function rather than its composition might help to understand its impact on the host and brain disease development. Although an increased stroke incidence in young individuals has been recently reported,[38] stroke risk factors such as age and sex were not addressed in this study. Interestingly, recent findings have demonstrated that reproductively senescent rats significantly affect gut communities under stroke conditions,[32] and a young microbiome is neuroprotective when transferred to aged mice.[23] Whereas the impact of lifestyle factors (diabetes mellitus, high blood cholesterol levels, alcohol consumption, high fat diet, lack of exercise) on the gut microbiome and stroke outcome remains to be addressed.

In conclusion, our findings highlight the importance of the intestinal microbiome in the short- and long-term outcome of ischemic brain injury and raise the possibility that modulation of microbial metabolism could be a novel preventive strategy in patients at high risk for stroke

Acknowledgments

Dr Benakis contributed to study design, performed and/or contributed to all experiments, analyzed data, and wrote the manuscript. In some experiments, Dr Benakis was assisted by Dr Poon, J. Moore, M. Murphy, Dr Sita, and G. Racchumi. Dr Lane performed the open field test and analyzed the data. Dr Brea performed the FACS analysis. Dr Iadecola contributed to study design. J. Anrather formulated the original hypothesis, designed the study, analyzed the sequencing data, and wrote the manuscript together with Drs Benakis and Iadecola. All authors read and approved the manuscript.

Sources of Funding

The study was supported by the US National Institutes of Health (NIH) grants NS081179, NS094507 (J. Anrather) and NS34179 (Dr Iadecola and J. Anrather), the Feil Family Foundation (Dr Iadecola), and the Swiss National Science Foundation for Grants in Biology and Medicine (P3SMP3 148367; Dr Benakis).

Disclosures

Dr Iadecola served on the Strategic Advisory Board and Broadview Ventures. The other authors report no conflicts.

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