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Microbiome-derived carnitine mimics as previously unknown mediators of gut-brain axis communication

Heather Hulme1, Lynsey M. Meikle1, Nicole Strittmatter2, Justin J. van der Hooft2, John Swales2, Ryan A. Bragg4, Victor H. Villar5, Michael J. Ormsby1, Stephanie Barnes6, Sheila L. Brown7, Alex Dexter5, Maya T. Kamat1, Jasper C. Komen9, Daniel Walker1, Simon Milling1, Emily K. Osterweil5, Andrew S. MacDonald7, Chris J. Schofield10, Saverio Tardito5,11, Josephine Bunch8,12, Gillian Douce1, Julia M. Edgar1,13, RuAngelie Edrada-Ebel14, Richard J. A. Goodwin2, Richard Burchmore1, Daniel M. Wall1*

Alterations to the gut microbiome are associated with various neurological diseases, yet evidence of causality and identity of microbiome-derived compounds that mediate gut-brain axis interaction remain elusive. Here, we identify two previously unknown bacterial metabolites 3-methyl-4-(trimethylammonio)butanoate and 4-(trimethylammonio)pentanoate, structural analogs of carnitine that are present in both gut and brain of specific pathogen-free mice but absent in germ-free mice. We demonstrate that these compounds are produced by anaerobic commensal bacteria from the family Lachnospiraceae (Clostridiales) family, colocalize with carnitine in brain white matter, and inhibit carnitine-mediated fatty acid oxidation in a murine cell culture model of central nervous system white matter. This is the first description of direct molecular inter-kingdom exchange between gut prokaryotes and mammalian brain cells, leading to inhibition of brain cell function.

INTRODUCTION

While the microbiome can exert a degree of protection against invading pathogens, substantial and lasting changes in its composition are linked to many diseases that until recently were thought to be independent of microbial influence. Recent work has demonstrated that the microbiome is also fundamentally changed in many neurological conditions including multiple sclerosis, Parkinson’s disease, autism spectrum disorders (ASDs), Alzheimer’s disease, and chronic fatigue syndrome (1, 2). Although little conclusive evidence supports a causative role for the microbiome in neurological disease, bidirectional communication between the gut microbiome and the brain is now recognized as an important mediator of neurological health. Studies in animal models underscore the importance of a stable microbiome, as its disruption, particularly at key developmental stages, results in long-term effects on anxiety, development, and behavior (3). In addition, positive effects of gut bacterial supplementation on the brain are reported, while the positive outcomes of therapeutic interventions in some cases can be attributed to the gut microbiota (4).

Microbes stimulate host neurotransmitter production in the gut and synthesize their own host-recognized neurotransmitters (5, 6). They can also stimulate the host immune system, leading to inflammation and notable shifts in composition of the gut microbiota with the potential to exert neurological effects (7). While microbial-derived molecules such as short-chain fatty acids can cross into the brain to exert their effects, most microbial molecules that influence the microbiome-gut-brain (MGB) axis mediate their effects indirectly through the highly enervated gut and the vagus nerve (8, 9).

RESULTS

To identify unknown microbial metabolites that mediate MGB axis communication, we used mass spectrometry imaging (MSI) to identify molecules that are present in both the gut and brain of specific pathogen–free (SPF) C57BL/6 mice but are absent from germ-free (GF) C57BL/6 mice. This approach enables the identification of both known and novel molecules, with high spatial resolution. We detected a molecule at a mass-to-charge ratio (m/z) of 160.133 in GF mice was comparable to a baseline off tissue negative control (Fig. 1 and fig. S1). MALDI-MSI was used to gain a high lateral resolution image for brain communication, we used mass spectrometry imaging (MSI) to identify molecules that are present in both the gut and brain of specific pathogen–free (SPF) C57BL/6 mice but are absent from germ-free (GF) C57BL/6 mice. This approach enables the identification of both known and novel molecules, with high spatial resolution. We detected a molecule at a mass-to-charge ratio (m/z) of 160.133 in GF mice was comparable to a baseline off tissue negative control (Fig. 1 and fig. S1). MALDI-MSI was used to gain a high lateral resolution image for brain region analysis, and DESI-MSI was used for further high spectral resolution analysis. The detection of the molecule at m/z 160.133 in GF mice was comparable to a baseline off tissue negative control (Fig. 1). This molecule, provisionally termed Met1, was particularly abundant in white matter areas of the brain (medulla, corpus callosum, and the arbor vitae) (Fig. 1). Met1 was also detected systemically in wild-type C57BL/6 mice, in blood, liver, kidney, lung, spleen, intestine, testes, and heart (fig. S2).
As detection of Met1 in GF mice was comparable to the negative control, this suggested a microbial origin for the compound in SPF mice. Therefore, we determined whether Met1 in the colon could be reduced by treatment for 7 days with a cocktail of nonabsorbable antibiotics. This treatment was seen to significantly reduce Met1 levels ($P \leq 0.05$), underscoring the likely microbial origin of Met1 (fig. S3). We next screened intestinal bacterial strains from the gut microbiota of C57BL/6 mice for the production of Met1. Bacterial strains isolated from murine feces were grown on fastidious anaerobe broth (FAB) agar plates before colonies were resuspended in phosphate-buffered saline (PBS) and spotted onto glass slides for screening for Met1 by MSI. A peak at $m/z$ 160.133 was detected in two closely related bacterial strains from the Clostridium XIVa cluster of the Lachnospiraceae family, determined by 16S ribosomal DNA (rDNA) sequencing to be \textit{Clostridium clostridioforme} and \textit{Clostridium symbiosum} (Fig. 2). Both strains are enteric spore-forming human gut commensals that are obligately anaerobic, while \textit{C. clostridioforme} is also an opportunistic pathogen (10).

Other clostridial strains tested here did not produce this molecule, and its production was not detected in all \textit{C. clostridioforme} strains tested, including the \textit{C. clostridioforme} type strain NCTC11224 (Fig. 2). To ensure that the bacterial metabolite was Met1, rather than a structural isomer, we carried out tandem mass spectrometry (MS/MS) analysis of the bacterial metabolite at $m/z$ 160.133 from \textit{C. clostridioforme} and Met1 from the murine brain. The Met1 product ion spectra from the different sources matched, confirming that the molecules from the murine brain and \textit{C. clostridioforme} were structurally identical (fig. S4). On the basis of the acquired MS/MS spectra, we concluded that Met1 is similar in structure to carnitine, as two indicative mass fragments for a trimethylamine group were observed ($m/z$ 58.066 and $m/z$ 60.081) along with the mass difference of a carboxylic acid group (the difference between product ions at $m/z$ 101.060 and $m/z$ 55.055). Furthermore, the MS/MS spectra generated appeared highly similar to that previously reported for 5-aminovaleric acid betaine (5-AVAB; also known as N-trimethyl-5-aminovalerate), which has an identical $m/z$ 160.133 (11). However, during MS/MS fragmentation, we noted distinct differences in the intensity of the peaks at $m/z$ 60.081 and $m/z$ 55.055 at higher collision energies between Met1 and a synthesized 5-AVAB standard, leading us to doubt that Met1 was 5-AVAB (fig. S5). To definitively elucidate the structure, we subjected the bacterial fraction containing Met1 to nuclear magnetic resonance (NMR) correlation spectroscopy ($^{1}H$-$^{1}H$ COSY).
Two microliters was allowed to air-dry on a slide before MSI. GLM31, (NCTC 11224 (type strain), JYM13, (faecalis Escherichia coli LM19R, and (control, F18. C. symbiosum).

NMR determined that Met1 was a mixture of two structural isomers differing only in the location of a methyl side chain, located on either C3 [3-methyl-4-(trimethylammonio)butanoate (3M-4-TMAB)] or C4 [4-(trimethylammonio)pentanoate (4-TMAP)] of the structure (Fig. 3). NMR analysis also showed that the metabolites were produced in an equimolar ratio by the bacteria alongside the congener molecule γ-butyrobetaine (GBB). Detection of GBB alongside 3M-4-TMAB and 4-TMAP in C. clostridioforme suggests that it may be the precursor of these molecules, as well as being the direct precursor of carnitine. However, unlike 3M-4-TMAB and 4-TMAP, we tested their ability to inhibit mitochondrial function in host cells. Using synthetic 3M-4-TMAB and 4-TMAP, we tested their ability to inhibit mitochondrial function in the presence of carnitine in a primary murine cell culture model of the central nervous system (CNS) white matter (13) using the XF palmitate–bovine serum albumin (BSA) FAO substrate on a Seahorse analyzer (Seahorse Bioscience). This assay measures FAO using the oxygen consumption rate (OCR) as a readout, therefore allowing us to evaluate whether a molecule is capable of inhibiting FAO. Murine spinal cord–derived cells were grown for 8 days in vitro before being assayed in the presence of 0.5 mM carnitine and 3M-4-TMAB and 4-TMAP, either individually or in combination, at 2 mM for 24 hours. FAO was significantly inhibited by 3M-4-TMAB alone (reduced by 12% across four biological replicates), by 4-TMAP (14% reduction), or by an equimolar mixture of 3M-4-TMAB/4-TMAP (17% reduction) (Fig. 6). The inhibition seen is comparable to that for etomoxir (25% reduction), an irreversible inhibitor of FAO. In addition, testing 3M-4-TMAB and 4-TMAP in combination at 20 μM, in the absence of carnitine supplementation into culture and assay media, indicated that 3M-4-TMAB and 4-TMAP, at concentrations similar to those found in the murine brain, could significantly reduce FAO in white matter cells (Fig. S8).

**Fig. 2.** DESI-MSI was used to screen intestinal bacteria for the production of metabolites of interest. Bacterial cultures were grown on solid FAB agar, and colonies were resuspended in PBS to OD600 (optical density at 600 nm) of approximately 0.3. Two microliters was allowed to air-dry on a slide before MSI. Bacterial strains tested: (A) C. symbiosum LM19R, (B) C. symbiosum LM19B, (C) C. clostridioforme LM41A, (D) C. symbiosum LM42D, (E) Bifidobacterium animalis LM33, (F) Lactobacillus animalis LM31, (G) Propionibacterium spp. YM23, (H) Clostridium difficile LM27, (I) Enterococcus faecalis YM13, (J) Bacteroides fragilis NCTC 9343 (type strain), (K) C. clostridioforme NCTC 11224 (type strain), (L) C. clostridioforme NCTC 7155 (type strain), (M) blank control, (N) blank control, (O) C. symbiosum LM19R, and (P) Escherichia coli f18.

NMR determined that Met1 was a mixture of two structural isomers differing only in the location of a methyl side chain, located on either C3 [3-methyl-4-(trimethylammonio)butanoate (3M-4-TMAB)] or C4 [4-(trimethylammonio)pentanoate (4-TMAP)] of the structure (Fig. 3). NMR analysis also showed that the metabolites were produced in an equimolar ratio by the bacteria alongside the congener molecule γ-butyrobetaine (GBB). Detection of GBB alongside 3M-4-TMAB and 4-TMAP in C. clostridioforme suggests that it may be the precursor of these molecules, as well as being the direct precursor of carnitine. However, unlike 3M-4-TMAB and 4-TMAP, we tested their ability to inhibit mitochondrial function in host cells. Using synthetic 3M-4-TMAB and 4-TMAP, we tested their ability to inhibit mitochondrial function in the presence of carnitine in a primary murine cell culture model of the central nervous system (CNS) white matter (13) using the XF palmitate–bovine serum albumin (BSA) FAO substrate on a Seahorse analyzer (Seahorse Bioscience). This assay measures FAO using the oxygen consumption rate (OCR) as a readout, therefore allowing us to evaluate whether a molecule is capable of inhibiting FAO. Murine spinal cord–derived cells were grown for 8 days in vitro before being assayed in the presence of 0.5 mM carnitine and 3M-4-TMAB and 4-TMAP, either individually or in combination, at 2 mM for 24 hours. FAO was significantly inhibited by 3M-4-TMAB alone (reduced by 12% across four biological replicates), by 4-TMAP (14% reduction), or by an equimolar mixture of 3M-4-TMAB/4-TMAP (17% reduction) (Fig. 6). The inhibition seen is comparable to that for etomoxir (25% reduction), an irreversible inhibitor of FAO. In addition, testing 3M-4-TMAB and 4-TMAP in combination at 20 μM, in the absence of carnitine supplementation into culture and assay media, indicated that 3M-4-TMAB and 4-TMAP, at concentrations similar to those found in the murine brain, could significantly reduce FAO in white matter cells (Fig. S8).
The application of a multidisciplinary approach and novel imaging methodologies here has enabled the elucidation of molecular mimicry as another means of communication across the MGB axis. Microbe-mitochondria cross-talk may be an important means of gut microbiome–host communication, and its biological importance in diseases where mitochondrial dysfunction and the gut microbiome are significant factors is likely underappreciated.

**DISCUSSION**

Alterations in the gut microbiome have been linked to a number of neurological conditions, meaning that understanding the means of microbial communication across the MGB axis is of increasing importance. This study identifies two novel gut microbiome–derived carnitine mimics that significantly colocalize with carnitine in the white matter of the murine brain while also inhibiting carnitine function in in vitro models of murine CNS white matter. FAO supplies up to 20% of brain oxidative energy needs and is critical for many neurological functions, with neural stem cells reliant in vitro and in vivo on FAO for their survival and proliferation (14, 15). Given recent findings underlining the fundamental importance of FAO to brain health, the identification of gut microbiome–derived compounds in the brain that mediate inhibition of brain cell function is of immense importance (15, 16).

Inhibition of mitochondrial function by antimicrobials has been reported previously, and there is a growing body of evidence to support the existence of microbiome-mitochondria communication within the host (17). While carnitine is not needed for fatty acid trafficking in microbes, it acts as both a nutrient source and a protectant against environmental stress (18). Carnitine metabolism by enteric microbes generates trimethylamine N-oxide (TMAO), high serum levels of which are indicative of cardiovascular disease risk (19). Intestinal bacteria can thrive in the presence of carnitine, but *Lachnospiraceae*, of which the 3M-4-TMAB– and 4-TMAP–producing *Clostridia XIV* are members,
decrease in its presence likely as a result of being outcompeted by carnitine-metabolizing bacteria (19). Under such competitive conditions, we speculate that carnitine mimics such as 3M-4-TMAB and 4-TMAP may be produced by these Lachnospiraceae in an attempt to inhibit carnitine function or metabolism in competing bacteria.

C. clostridioforme and C. symbiosum, the identified producers of 3M-4-TMAB and 4-TMAP, remain poorly understood. C. clostridioforme is now recognized as distinct from C. bolteae and C. hathewayi, two species that were previously classified as C. clostridioforme (10). C. bolteae rapidly proliferates after disruption of the human gut microbiota, likely because of its ability to form endospores and resistance to multiple antibiotics, traits also found in C. clostridioforme and C. symbiosum (20, 21). C. bolteae exhibits a marked ability to proliferate and persist in the human intestine post-antibiotic use, maintaining its presence for 180 days after treatment (20). The presence of C. symbiosum and C. clostridioforme in the gut microbiome is associated with low microbial diversity, while C. symbiosum presence could further distinguish obese from lean participants (22). The link between C. symbiosum and metabolism is further underlined by its ability to promote robust weight gain in growth-stunted mice, one of only two bacteria that exhibited the capability (23). Whether 3M-4-TMAB and 4-TMAP could play any role in these metabolic effects warrants further investigation, but we hypothesize that under conditions that are favorable to increased colonization by these microbes, the production of 3M-4-TMAB and 4-TMAP in the intestine is likely to increase significantly with potential systemic effects.

While 3M-4-TMAB is a molecule not previously described in the literature, 4-TMAP has been chemically synthesized as an analog of mildronate, a potent inhibitor of both human GBB hydroxylase (BBOX1) and carnitine acetyltransferase (CrAT) enzymes (24). These enzymes are involved in carnitine synthesis and the transport of fatty acids into mitochondria, respectively. 4-TMAP is bound in the human BBOX1 active site in a near-identical conformation to mildronate and has a lower dissociation constant (4.1 μM) but was found to have an inhibitory concentration (IC50) nearly three orders of magnitude higher than mildronate (24–26). This means that the inhibitory effects seen here are likely mediated by targeting of carnitine-related enzymes other than BBOX1 and CrAT. 3M-4-TMAB bears marked structural similarity to a number of known inhibitors of carnitine function, including aminocarnitine derived from the fungal compound emericidin (27). Aminocarnitine inhibits carnitine palmitoyltransferase 1 (CPT1) and CPT2, and CrAT as the hydroxyl group essential to carnitine metabolism has been substituted with an alternate nonfunctional side chain. Removal of this hydroxyl group, which is also absent in 3M-4-TMAB and replaced with a methyl group, prevents the esterification reaction needed to link a long-chain fatty acid to carnitine, thereby inhibiting fatty acid shuttling into the mitochondria for oxidation. Future research focusing on a role for metabolites...
Mitochondrial dysfunction is also common across a range of neurological conditions, with neuronal cells highly dependent on dynamic mitochondria to meet their high energy requirements (16, 36, 37). A number of ASDs have been linked to underlying mitochondrial dysfunction, with descriptions in the literature describing increased acylcarnitine levels, decreased FAO-mediated stem cell generation, and carnitine-mediated improvement in symptoms (16, 38). While the significance of alterations in the gut microbiota in ASD remains controversial, both of the 3M-4-TMAB/4-TMAP–producing strains identified here have been described in elevated numbers in the gut while being at low levels or completely absent in controls (39). These microbiome changes are mirrored in murine ASD models such as the maternal immune activation (MIA) model, with increases in Lachnospiraceae again noted (4). After treatment decreased Lachnospiraceae numbers in the intestine of the MIA model, improvements in communicative, stereotypic, anxiety-like, and sensorimotor behaviors were all noted, along with a correction in defective fatty acid metabolism. These mitochondrial deficiencies, alongside those described in other neurological conditions such as Parkinson's and Alzheimer's disease, mean that any potential role for microbiome input into mitochondrial inhibition is worthy of further investigation.

This is the first mechanistic description of a microbial molecule inhibiting the function of the mitochondria in cells of the CNS. The two novel molecules produced by the gut microbiome described here are the first found in the murine brain that localize with and antagonize the function of carnitine. Given their potency at the physiological concentrations found in the murine brain, our findings indicate that neurological conditions, where mitochondrial dysfunction has been described and where disturbances in the gut microbiome are noted, should be looked at with increased emphasis on potential for microbiome input.

** MATERIALS AND METHODS**

**Animal work**

Seven- to 8-week-old male C57BL/6J GF and SPF mice were sourced from the University of Manchester, Gnotobiotic Facility. Both GF and SPF mice were fed the same pelleted diet, which was sterilized by irradiation with 50 kGy. The Manchester Gnotobiotic Facility was established with the support of the Wellcome Trust (097820/Z/11/B) using founder mice obtained from the Clean Mouse Facility, University of Bern. For determination of bacterial metabolites in organs other than the brain and to determine the effects of antibiotic treatment on the production of 3M-4-TMAB/4-TMAP in the intestine, C57BL/6J mice that were age- and sex-matched to GF mice were sourced from the University of Glasgow. For antibiotic treatment, mice were administered gentamicin (1 mg/ml), neomycin (1 mg/ml), and vancomycin (0.5 mg/ml) in sterile distilled drinking water for 7 days. Approval for these procedures was given before their initiation by internal University of Manchester and University of Glasgow ethics committees and the U.K. Home Office under licenses 70/7815, P64BCA712, and P78DD6240.

**Tissue processing**

Mice were culled by cervical dislocation, and brains and colons were immediately dissected. The colons were cut along the length, fecal matter was removed, and the colon was rolled and embedded in 2.5% carboxymethyl cellulose (CMC) (Sigma-Aldrich). Brains were placed unembedded in a mold before freezing using crushed dry ice and...
ethanol. The testis, heart, lung, liver, kidney, spleen, and mesenteric lymph nodes were collected from one C57BL/6J mouse, embedded in 2.5% CMC and snap-frozen in crushed dry ice and ethanol. Blood was collected by cardiac puncture and spotted onto a glass slide and allowed to air dry. Both brains and colons were cut using a cryostat microtome (Leica) at 10 μm thickness at −18°C, and the sections were thaw-mounted onto indium tin oxide–coated slides for MALDI-MSI and normal glass slides for DESI-MSI. Consecutive brain and gut sections next to those taken for MSI were used for hematoxylin and eosin staining. All slides were stored at −80°C until analysis.

Matrix application and MALDI-MSI analysis
MALDI was used for the initial analysis to obtain a high lateral resolution image to show an accurate distribution of the metabolite in the brain. Extensive further analysis was performed using high mass resolution DESI analysis for MS/MS experiments, showing that the spectra from the metabolites in the SPF brain and the spectra produced by the bacteria are highly similar. The increase in mass resolution is demonstrated in fig. S9. MSI of brains and guts from GF and SPF mice was carried out by MALDI–time-of-flight (TOF). The results were confirmed by MSI with a DESI-orbitrap to gain a more accurate m/z value. Before matrix application, the slide was taken from −80°C and brought to room temperature under a stream of air.

DESI-MSI analysis
DESI-MSI was performed on an orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific) equipped with an automated Prosolia 2D DESI source (Prosolia OmniSpray 2D). The DESI source was modified with the following parameters. The spray tip was positioned at 1.5 mm above the sample surface and at an angle of 75°. The distance between the sprayer to mass spectrometer inlet was 7 mm with a collection angle of 10°. The spray solvent was methanol/water (95:5, v/v), delivered at 1.5 μl/min using a Dionex Ultimate 3000 pump (Thermo Fisher Scientific) at a spray voltage of 4.5 kV. Nitrogen was used as the nebulization gas at a pressure of 160.01 and 162.01 (carnitine) were extracted and segmented into m/z 160.133 with single isolated colonies of murine gut microbiome bacteria or type strains were grown overnight in FAB or on FAB agar. Taxonomic assignment for the strains was obtained using the complete 16S rDNA sequence from each strain and the “Identify” function in EzBioCloud (41). The 3M-4-TMAB/4-TMAP–producing strain, designated LM19, was identified as C. symbiosum, being 99.79% similar to the C. symbiosum type strain ATCC14940. The second producing strain, designated LM41, was identified as being 99.5% similar to the C. clostridioforme type strain ATCC25537. Type strains used for MSI were from the National Collection of Type Cultures (United Kingdom). For MSI analysis, single colonies from FAB agar plates were inoculated into 100 μl of PBS to give an optical density at 600 nm of 0.3. Two microliters of each bacterial culture was spotted onto a glass slide and allowed to dry. Slides were stored in a desiccator until DESI-MSI, which was performed using the same DESI-MSI parameters as above with a spatial resolution of 200 μm.

Nuclear magnetic resonance
Samples (15 mg) were dissolved in 700 μl of deuterated water (Sigma-Aldrich) and added to a 7-mm NMR tube. Presaturation proton NMR experiments were performed by irradiating the water peak at 4.88 parts per million (ppm), using 128 scans, 15-Hz spin with a relaxation delay of 3 s. NMR was carried out using a 400-MHz JEOL LA400 FT-NMR spectrometer system equipped with a 40TH5AT/FG probe (JEOL, Tokyo, Japan). The acquisition of the one-dimensional proton spectra (1H NMR) was performed by the presaturation pulse sequence using 128 scans per analysis. Subsequently, two-dimensional COSY NMR spectra were also acquired for 64 scans. Data points were collected into a plot using spectral widths of 3.93 kHz for F1 and F2. The presaturation method was used to suppress the solvent signal during acquisition.

Analysis of correlation of m/z 160.133 with carnitine in the brain
Data were first converted into imzML using flexImaging (Bruker Daltonics version 4.1), and processing was performed using SpectralAnalysis. Peaks were picked from a mean spectrum generated without any preprocessing using gradient peak detection, and a data cube was generated by integrating the area under each peak. All data were then exported into MATLAB (version 2017a and statistics and image processing toolbox, The MathWorks Inc., Natick, MA) for further analysis.

Tissue data were first segmented from background using k-means clustering (k = 2, cosine distance). Following this, the images from m/z 160.01 and 162.01 (carnitine) were extracted and segmented into binary masks of high and low intensity using a k-means clustering (k = 2, Euclidean distance). The overlap of these regions was then calculated as the percentage of pixels in the high-intensity region of the m/z 160.1 image that were also high intensity in the m/z 162.1 image. In addition, Pearson’s correlation coefficient between the two images was calculated for each tissue.

Synthesis of 3M-4-TMAB and 4-TMAP
All amino acid starting materials were from UkrOrgSyntez Ltd. The general procedure of Lukevics was used to prepare the materials (42). The method of Tars was used for purification (24).

4-TMAP [rac-4-(trimethylammonio)pentanoate] methyl-N,N'-diisopropylcarbamimide (0.500 ml, 2.75 mmol) was added dropwise to a stirred solution of 4-(dimethylamino)pentanoic acid

hydrochloride (250 mg, 1.38 mmol) in MeOH (4 ml) at 21°C over a period of 1 min under nitrogen. The resulting solution was stirred at 21°C for 40 hours. The reaction mixture was concentrated to dryness under reduced pressure, and the residue was slurried in water (6 ml) for 1 hour. The precipitated urea was removed by filtration, and the mixture was evaporated to dryness to give a gum. This material was dissolved in water (5 ml) and loaded onto a column packed with 5 ml of Amberlite IRN78 hydroxide form resin, and the column was stoppered for 30 min before being eluted with water. Product-containing fractions were combined and evaporated under reduced pressure, and the resulting residue was evaporated from acetonitrile to give rac-4-(trimethylammonio)pentanoate (204 mg, 1.281 mmol, 93%) as a waxy solid. 1H NMR (500 MHz, d6-DMSO) 1.23 (d, J = 6.54 Hz, 3H), 1.29 to 1.39 (m, 1H), 1.76 (ddd, J = 6.0, 9.5, 15.5 Hz, 1H), 1.93 (ddd, J = 5.5, 6.45, 15.0 Hz, 1H), 2.1 to 2.18 (m, 1H), 2.98 (s, 9H), and 3.43 (ddq, J = 2.5, 6.5, 10.5 Hz, 1H); 13C NMR (126 MHz, D2O, 27°C) 180.9, 71.0, 50.6, 34.2, 26.4, and 12.7; liquid chromatography (LC–MS): m/z 160.1. Extracted mean relative abundance was used from each area of interest to construct calibration curves in Microsoft Excel using a linear fit with unknown endogenous concentrations calculated using the equation of a straight line.

**Cell culture**

CNS myelinating cultures were established as described (13) with minor modifications. Briefly, embryonic day 13 (E13; day of plug E0) mouse spinal cords were isolated and stripped of their meninges and then dissociated into a single-cell suspension using trypsin and trituration. Cells were plated at 60,000 to 75,000 cells per well of a Seahorse XF96 cell culture plate (35-μl volume) pretreated with poly-l-lysine [in boric acid buffer (0.1 mg/ml; pH 8.4)]. Cells were plated initially in 12.5% horse serum, which was gradually withdrawn through feeding every second or third day with serum-free differentiation medium [Dulbecco’s modified Eagle’s medium (DMEM) (glucose, 4.5 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), biotin (10 ng/ml), 1% N1, 50 mM hydrocortisone, and insulin (10 μg/ml)]. Cells were maintained in 5% CO2 at 37°C.

**OCR measurements**

On day in vitro (DIV) 8 for CNS myelinating cultures, OCR was measured using the XF Cell Mito Stress Test and XF Palmitate-BSA FAO substrate on a Seahorse XF96 analyzer (Seahorse Bioscience, Billerica, MA). Twenty-four hours before the assay, cultures were placed in Substrate-Limited Medium [DMEM supplemented with 0.5 mM glucose, 1 mM glutamine, 0.5 mM carnitine, and 1% fetal bovine serum (FBS)]. Just before the assay, media were changed to FAO Assay Medium consisting of KHB buffer (111 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl2, 2 mM MgSO4, and 1.2 mM Na2HPO4) supplemented with 2.5 mM glucose, 0.5 mM carnitine, and 5 mM Hepes, adjusted to pH 7.4. To stimulate FAO, palmitate-BSA substrate (200 μM) was added to all wells except the BSA control. 4-TMAP and 3M-4-TMAB effects on FAO were measured by supplementation into wells of 2 mM 4-TMAP or 3M-4-TMAB or 1 mM 4-TMAP and 3M-4-TMAB in combination for 24 hours before the assay and for the duration of the assay. For examination of the effect of the concentrations of 3M-4-TMAB and 4-TMAP found in the brain on FAO, the experiment was repeated as before but in the absence of carnitine (0.5 mM) supplementation into both Substrate-Limited Medium and FAO Assay Medium, meaning that FBS was the only exogenous source of carnitine during the assay. 4-TMAP or 3M-4-TMAB (20 μM), or a combination of 10 μM 4-TMAP and 10 μM 3M-4-TMAB, was supplemented into medium for 24 hours before assay and during the assay. OCR was measured over time in the presence of either high or low concentrations of 3M-4-TMAB and 4-TMAP, and at least three repeated measures per well were recorded. For each well, the measurements were normalized on the total micrograms of proteins determined at the end of the assay with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). The number of replicate wells varied between 5 and 20 per condition. The values of the repeated measurements were averaged, and a mean value was obtained for each individual well. The OCR values per well were further averaged, and a mean value for each condition was obtained. Last, the mean value for each of the conditions was divided by the value obtained for the condition with palmitate and BSA supplementation. These calculations were performed using mass selection window of ±0.05 Da. 3M-4-TMAB/4-TMAP was detected as the protonated molecular ion (M+H+) at m/z 160.1. Extracted mean relative abundance was used from each area of interest to construct calibration curves in Microsoft Excel using a linear fit with unknown endogenous concentrations calculated using the equation of a straight line.
applied to each of the independent experiments performed, for which the relative OCR values are reported in the figure.

**Microbiome analysis of antibiotic-treated mice**

For preparation of DNA samples for microbiome sequencing, fecal samples were defrosted to room temperature and the FastDNA Spin Kit for Soil DNA Extraction (MP Biomedicals) was used for DNA extraction; 16S ribosomal RNA (rRNA) gene sequencing was undertaken at Glasgow Polymics for analysis of the microbiome. In brief, primers that were specific to the V3 and V4 regions were used to generate 16S primer sequences from high-throughput sequencing reads with a minimum quality of 25 and a minimum length of 250 bp. Paired-end reads were completed using the Qiime wrapper and software.

Cutadapt removes adapter barcodes and adapters added using the Nextera XT v2 adapter sets. Further processing and analysis were performed using the Qiime wrapper and software.

**Statistical analysis**

For analysis of FAO, at least three independent experiments were undertaken, with each biological replicate shown. For each of the conditions tested, the value of OCR was expressed as relative to the control area.

**REFERENCES AND NOTES**


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Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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