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2 **Mapping the influence of the gut microbiota on small molecules in the brain**
 3 **through mass spectrometry imaging**

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27 **Abstract**

28 **Background:** The gut microbiota is known to influence virtually all facets of human health.

29 Recent work has highlighted a potential role for the gut microbiota in neurological health
30 through the microbiome-gut-brain axis. Microbes can influence the brain both directly and
31 indirectly; through neurotransmitter production, induction of host immunomodulators, or
32 through the release or induction of other microbial or host molecules.

33 **Methods:** Here we used mass spectrometry imaging (MSI), a label-free imaging tool, to map
34 the molecular changes that occur in the murine gut and brain in germ-free, antibiotic-treated
35 and control mice.

36 **Results:** We determined the spatial distribution and relative quantification of
37 neurotransmitters and their precursors across brain and gut sections in response to the
38 microbiome. Using untargeted MSI of small molecules, we detected a significant change in
39 the levels of four identified metabolites in the brains of germ-free animals compared to
40 controls; vitamin B5, 3-hydroxy-3-methylglutaric acid, 3-methyl-4-
41 (trimethylammonio)butanoate and 4-(trimethylammonio)pentanoate. However, antibiotic
42 treatment induced no significant changes in these metabolites in the brain after one week of
43 treatment.

44 **Conclusions:** This work exemplifies the utility of MSI as a tool in determining the spatial
45 distribution and quantification of bacterial and host metabolites in the gut and brain whilst also
46 offering the potential for discovery of novel mediators of microbiome-gut-brain axis
47 communication.

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49

50

51 Background

52 Deciphering the complex bi-directional communication across the microbiome gut brain
 53 (MGB) axis remains a challenging prospect. The composition and stability of the gut
 54 microbiome is now proposed to be a significant contributor to human health with changes in
 55 its composition suggested as a contributing factor in a number of neurological conditions.
 56 Diverse human neurological disorders, ranging from autism spectrum disorders (ASDs) and
 57 attention-deficit/hyperactivity disorder (ADHD) to Alzheimer's and Parkinson's disease, have
 58 linked gastrointestinal abnormalities or changes in the gut microbiome (1–6). Similarly,
 59 altered levels of certain bacterial species in the gut have been linked with depression (7, 8).
 60 Despite the deficiencies of germ free (GF) animal models, their use for investigating links
 61 between the gut microbiota and the brain have proved informative and are helping to uncover
 62 the influence of bacteria on neurotransmitter levels alongside other bacterial and host
 63 metabolites. Their neuro-developmental abnormalities including increased blood-brain-barrier
 64 (BBB) permeability, alterations in abundance and maturity of microglia cells, and reduced
 65 myelination (9–11) have been well documented but their use, alongside antibiotic treated
 66 (ABX) mice, have provided further evidence that supports the importance of a stable and
 67 healthy gut microbiota in maintaining normal cognitive function and development (12). GF
 68 mice display reduced anxiety-like behaviour and an increased response to stress that is fully
 69 alleviated upon colonisation with *Bifidobacterium infantis* (12–15) while murine ABX treatment
 70 models reinforce the significance of the MGB axis with treated mice showing impaired
 71 cognition and significantly altered behaviours that can be linked to absence of particular
 72 bacteria (16–19). These effects are likely mediated by the numerous bacterial molecules
 73 including short chain fatty acids (SCFAs), phenolic acids, quorum sensing peptides,
 74 peptidoglycan and lactic acid, that have been proposed to exert influence across the MGB
 75 axis (20–22). SCFAs both cross and modulate the permeability of the BBB, while animals and
 76 humans are also dependent on intestinal microbes to produce or supplement their vitamin
 77 needs (10, 23, 24).

78 Signalling across the MGB axis can occur via the vagus nerve, bacterial modulation of host
79 immune responses or host neurotransmitter production, and signalling through bacterial
80 neurotransmitters or other microbial molecules. Microbes produce a number of
81 neurotransmitters including gamma aminobutyric acid (GABA), norepinephrine and
82 dopamine, serotonin, and acetylcholine while bacterial production or depletion of essential
83 neurotransmitter intermediates such as tryptophan can also affect host production (14, 19,
84 25–30). Manipulation of the microbiome can also alter host neurotransmitter levels as
85 microbes stimulate their intestinal production with depletion of the microbiome, or its
86 supplementation with probiotic bacteria, capable of altering specific levels of
87 neurotransmitters even in the brain (31–35).

88 Understanding how the gut microbiota influence the brain, and the complex network of
89 molecules and neurotransmitters that mediate this influence, is a significant challenge
90 requiring novel tools and approaches. Mass spectrometry imaging (MSI) is a molecular
91 imaging tool that can be applied to understand and map biological systems with recent
92 successes in mapping microbial interactions with their environment (36–38). MSI maps the
93 distribution of small molecules across a tissue section independent of any label, so no prior
94 knowledge of the molecules present is required. The ability to detect spatial distribution and
95 abundance of thousands of compounds simultaneously in tissue sections makes this a
96 powerful approach for studying the MGB axis. Here, using MSI, we detected significant
97 differences in neurotransmitter levels and those of their precursors in the guts of both GF and
98 ABX mice. However in the brain no differences were detected in neurotransmitter levels in GF
99 mice compared to controls, while only tryptophan levels significantly changed in the brains of
100 ABX mice. Through untargeted MSI we also discovered 3 metabolites were significantly
101 changed in the GF brain. Two of these were identified as 3-hydroxy-3-methylglutaric acid (3-
102 HMG) and pantothenic acid (vitamin B5). 3-HMG, a metabolite associated with oxidative
103 stress, was significantly increased in the GF brain, while vitamin B5, which can be produced
104 by the microbiome, was present at a lower level in GF brain and is implicated in brain health.

105 This work indicates that the brain remains largely protected from microbiome changes in the
106 gut and indicates plasticity, not just in the brain but across the MGB axis in GF mice.

107

108 **Methods**

109 ***Animal models used in this study***

110 *Germ-free studies*

111 All germ-free (GF) work was undertaken at the University of Manchester Gnotobiotic Facility.
112 Five GF and specific pathogen free (SPF) mice were used in this study, which were male
113 mice, aged 7-8 weeks, on a C57BL/6J background strain. Both experimental groups were fed
114 the same pelleted diet that was irradiated with 50 kGy to ensure sterility. The Manchester
115 Gnotobiotic Facility was established with the support of the Wellcome Trust
116 (097820/Z/11/B), using founder mice obtained from the Clean Mouse Facility (CMF),
117 University of Bern, Bern, Switzerland.

118 *Antibiotic studies*

119 All work involving antibiotic (ABX) treatment was undertaken at the University of Glasgow.
120 Five ABX treated animals and corresponding untreated controls were used in these studies
121 and were male mice, aged 7-8 weeks, on a C57BL/6J background strain. The ABX cocktail
122 consisted of 1 mg/ml gentamicin, 1 mg/ml neomycin and 0.5 mg/ml vancomycin in sterile
123 distilled drinking. ABX supplemented drinking water was provided ad libitum for a period of 1
124 week and refreshed every 2 days. The untreated controls were given sterile drinking water
125 without ABXs ad libitum, which again was refreshed every two days. Both treated and control
126 groups were fed the same standard chow. All antibiotics selected for this study were chosen
127 based on their specificity for the gut microbiota; to the best of our knowledge the antibiotics
128 used are not absorbed by the intestine. Approval for these procedures was given prior to their
129 initiation by internal University of Manchester and University of Glasgow ethics committees
130 and by the U.K. Home Office under licenses 70/7815, PPL40/4500, P64BCA712 and
131 P78DD6240.

132

133 ***Tissue processing***

134 Mice were culled by cervical dislocation and brains and colons were removed. Brains were
135 placed in a mould and were immediately frozen using a slurry of dry-ice and ethanol to
136 maintain structural integrity and to ensure that all biochemical processes were halted. The
137 colon samples were then cut lengthwise over ice and the faecal matter was removed and the
138 remaining GI tissue was then rolled using the 'Swiss roll technique' before embedding in
139 2.5% medium viscosity carboxymethyl cellulose (Sigma-Aldrich, Dorset, UK). Both brains and
140 colons were sectioned using a CM3050S cryostat microtome (Leica Biosystems, Nussloch,
141 Germany) to 10 µm thickness at -18°C. Sagittal brain sections were either thaw mounted onto
142 indium tin oxide (ITO) coated slides for Matrix Assisted Laser Desorption/Ionization (MALDI)-
143 MSI or normal microscope slides for desorption electrospray ionisation (DESI)-MSI.
144 Stereotactically matched sections were selected to ensure that all comparisons were
145 performed using corresponding brain regions. Consecutive sections to those for MSI were
146 collected for histology purposes. Slides were prepared and stored at -80°C until required for
147 analysis. Prior to derivatisation, matrix application or analysis, the slide was taken from -80°C
148 and brought to room temperature under a stream of air.

149

150 ***Neurotransmitter derivatisation***

151 The derivatisation of primary amine neurotransmitters was performed as previously described
152 (39). Nine milligrams of 2,4-diphenyl-pyranilium tetrafluoroborate (DPP-TFB) was added to
153 1.2 mL of 100% methanol and sonicated for 20 minutes (min). This was then gradually added
154 to 6 mL of 70% methanol in water with 3.5 µL of trimethylamine. This solution was sprayed
155 onto the tissue for derivatisation using an automated sprayer (TM-Sprayer, HTX
156 Technologies); 30 passes were performed using a nozzle temperature of 75°C, velocity of
157 1100 mm/min, flow rate of 80 µL/min, and gas pressure of 6psi. After coating, the slide was

158 incubated in a petri dish with vapour from a 50 % methanol/water solution 3 times for 5
159 minutes each.

160

161 ***DESI-MSI analysis***

162 DESI-MSI was performed on a Q-Exactive mass spectrometer (Thermo Scientific, Waltham,
163 MA, US) equipped with an automated 2D DESI source (Prosolia Inc, Indianapolis, IN, USA).

164 A home-built DESI sprayer assembly, as described previously (40), was used with the spray
165 tip positioned at 1.5 mm above the sample surface and at an angle of 75°. The distance
166 between the sprayer to mass spectrometer inlet was 7 mm with a collection angle of 10° and
167 <<1mm distance between inlet and sample surface. The spray solvent was methanol/water
168 (95:5 v/v), delivered at 1.5 µL/min using a Dionex Ultimate 3000 pump (Thermo Scientific,
169 Waltham, MA, US) at a spray voltage of ±4.5 kV. Nitrogen was used as the nebulisation gas
170 at a pressure of 7 bars. General instrument settings used to image specific molecules are
171 shown in Table 1. For acquisition of MS/MS spectra, an injection time of 300 ms, mass
172 resolution of 70000 and a mass isolation window of ± 0.3 Da was used. For MS/MS imaging
173 of metabolites with mass to charge (*m/z*) ratios 161.044 and 218.103, various fragmentation
174 HCD settings were used, at a spatial resolution of 100 µm.

175 Data was recorded as individual line scans and converted into imzML format using imzML
176 converter version 1.1.4.5 (41) and visualised using MSiReader version 0.09 (42). All
177 intensities shown are raw ion intensities and 1st order linear interpolation was used for image
178 generation. All mean intensities of the molecules of interest were determined across the
179 entire tissue section. The data were transformed to log10 and a Shapiro-Wilk normality test
180 was performed on the data to check for a normal distribution. If the data passed the normality
181 test an unpaired t-test was performed. If the data failed the test a Mann-Whitney U test was
182 performed. When biological replicates were analysed over two separate DESI-MSI
183 experiments a paired t-test was performed instead.

184

185 *H&E staining of brains and colons*

186 Brain and colon sections that had undergone MSI analysis were H and E stained post-
 187 imaging to permit localisation of candidate metabolites and neurotransmitters to specific brain
 188 regions. Sections were fixed on the slide in ice cold 75% acetone and 25% ethanol for 10
 189 min, and air dry for a further 10 min. Slides were placed in water for 2 min, submerged in
 190 haematoxylin (Sigma-Aldrich, Poole, Dorset, UK) for 2 min and immediately rinsed in cold
 191 running water. The slides were then dipped for 3 sec in acid alcohol 0.5% (Atom Scientific
 192 Hyde, Cheshire, UK), and rinsed in water before submerging in Scott's tap water (Atom
 193 Scientific Hyde, Cheshire, UK) for a further 30 sec. The sections were counter-stained with
 194 eosin (Sigma Aldrich, Poole, Dorset, UK) for 2 min and washed in water. Sections were then
 195 dehydrated in increasing concentrations of ethanol (70 % ethanol for 30 sec, 90 % for 1 min,
 196 and twice for 3 min in 100 % ethanol), cleared in xylene (twice for 3 min), and cover slipped
 197 using DPX mounting media (Atom Scientific, Hyde, Cheshire, UK).

198

199 Results

200 ***Targeted neurotransmitters remain unaffected by the lack of a gut microbiota***

201 The brains and colons from germ free (GF) and conventionally colonized, specific pathogen
 202 free (SPF) control mice were first compared by MSI using a targeted approach. DPP-
 203 derivatisation of primary amines was performed to allow targeted imaging of
 204 neurotransmitters, neurotransmitter precursors and neurotransmitter metabolites; serotonin,
 205 tryptophan, dopamine, tyrosine, 3-methoxy-tyramine, GABA and glutamate (Figure 1) (39, 43,
 206 44). Significant differences in the levels of several neurotransmitters were observed in the
 207 gut. Serotonin was lower (3.8-fold decrease) in the GF mouse gut (Fig. 1b; $P \leq 0.01$), whilst
 208 glutamate (1.3-fold increase) (Fig. 1b; $P \leq 0.05$) and the dopamine precursor tyrosine (1.8-fold
 209 increase) (Fig. 1b; $P \leq 0.01$) were significantly increased in the GF gut. However, these
 210 changes were not reflected in the corresponding brain sections of GF mice where no
 211 significant differences were identified for any of the seven neurotransmitters or their
 212 precursors (Fig. 1a). There were also no differences detected in the levels of tryptophan,
 213 dopamine, 3-methoxy-tyramine or GABA in either the brain or gut sections from GF and SPF
 214 mice (Supplementary Figure 1).

215

216 ***MSI of neurotransmitters in the brains and colons of ABX treated mice***

217 To determine whether an acute disruption of the microbiota over 7 days could have an
 218 influence on the levels or the localisation of the same neurotransmitters in the brain, the colon
 219 and the brains of ABX treated mice were imaged in a targeted manner.
 220 Tyrosine levels were changed in ABX treated mice in a manner similar to that seen in GF
 221 mice with a significant increase in the colon (2.2-fold), but again this increase was not
 222 reflected in tyrosine levels in the brain of ABX treated animals (Fig. 1c and 1d; $P \leq 0.05$).
 223 Unlike GF mice, ABX treated mice showed no change in the levels of serotonin in either the
 224 colon or brain, compared to untreated mice (Fig. 1c and 1d). However, there was significantly
 225 higher abundance of tryptophan in both the colon (3.2-fold) ($P \leq 0.05$) and brain (1.7-fold) (P

226 ≤ 0.01) of ABX treated mice compared to controls (Fig. 1c and 1d). This was noted across the
 227 whole brain. Dopamine levels were also significantly higher (3.3-fold) in the colons from ABX
 228 treated mice compared to untreated, but no significant change was detected in the brain (Fig.
 229 1d; $P \leq 0.001$). No significant difference was detected in levels of 3-methoxytyramine,
 230 glutamate or GABA in either the gut or brain sections imaged (Supplementary Figure 2).

231

232 ***Untargeted MSI to detect novel molecular changes in GF mouse brains***

233 As no significant changes were seen in several neurotransmitters in GF brains, untargeted
 234 imaging was performed using DESI-MSI on the colon and brains of GF mice to probe the
 235 MGB axis for molecular changes induced by microbiota disruption. Full scan spectra were
 236 collected from m/z 65-400 and m/z 250-1000, in both positive and negative ionisation mode,
 237 allowing detection of a wide range of metabolites and not targeted towards a particular group.
 238 Significant differences were detected in only three masses when comparing GF and SPF
 239 mouse brains. Two of these identified metabolites at m/z of 218.1030 and m/z 161.0446, both
 240 detected in negative ion mode, were selected for further analysis as putative identities could
 241 be assigned from online databases, as discussed further below. The third mass at m/z
 242 160.133 was below the limit of detection in GF brains and could not be assigned an identity
 243 from online databases. Determination of the identity of this mass, the associated structures
 244 and bacterial origin has been recently described (Hulme *et al.*, In press).

245

246 ***Levels of 3-hydroxy-3-methylglutaric acid (3-HMG – m/z 161.0446) are increased in the*** 247 ***GF brain***

248 The molecule detected at m/z 161.0446 in negative ionisation mode, was found at
 249 significantly higher levels ($P \leq 0.0001$) across the whole brain section of GF compared to SPF
 250 mice, with levels particularly high in the cerebellum (Fig. 2a). The change in the brain was not
 251 reflected in the gut as levels in the colon did not significantly change between GF and SPF
 252 control mice (Fig. 2b). The metabolite was identified as [M-H]⁻ of 3-hydroxy-3-methylglutaric

253 acid (3-HMG) through searching the Human Metabolome Database (45, 46) and then
 254 confirmed using tandem mass spectrometry (MS/MS) analysis and comparison to an HMG
 255 standard (Supplementary figure 3). No significant difference in abundance of HMG was found
 256 in brains and colon after ABX treatment compared to controls (Fig. 2c and 2d).
 257 Quantification analysis of HMG revealed an average concentration of 0.56 µg/g of tissue in
 258 the GF brain compared to <0.01 µg/g of tissue in the SPF brain (Supplementary figure 4).
 259 The concentration of HMG was particularly high in the cerebellum in the GF mouse brain at a
 260 concentration of 5.02 µg/g of tissue compared to <0.01 µg/g of tissue in the SPF mouse
 261 brains.

262

263 ***Vitamin B5 (m/z 218.1030) levels are decreased in the brain of GF mice***

264 The second of the unknown molecules had an *m/z* of 218.1030 in negative ionisation mode
 265 and was found to be significantly lower in the brain of GF mice compared to SPF controls
 266 (Fig. 3a; *P* ≤ 0.05). This difference was most obvious in the cerebellum, the hippocampus and
 267 the hypothalamus, where the molecule was most abundant in the SPF mice. This molecule
 268 was unchanged between GF and SPF colons. The metabolite was identified as [M-H]⁻ of
 269 pantothenic acid, also known as vitamin B5, through database search and subsequent
 270 confirmatory MS/MS analysis.

271 No difference was found in the levels of vitamin B5 in the brain or colon in ABX treated mice
 272 compared to untreated control mice (Fig. 3a and 3b).

273 Quantification analysis of vitamin B5 revealed an average concentration of 0.25 µg/g of tissue
 274 in the GF brain compared to 0.36 µg/g of tissue in the SPF brain.

275

276 **Discussion**

277 Studying the MGB axis has significant potential to help us understand and potentially treat,
 278 via the microbiome, certain neurological conditions. However in order to achieve this goal a
 279 greater understanding of MGB communication is required. The techniques applied to date to

280 discover mediators of communication across the MGB axis have typically been targeted
 281 toward specific neurotransmitters, metabolites or regions of the brain (14, 19, 26). Such
 282 approaches are often dependent on analyte extraction from brain tissue prior to analysis,
 283 meaning data regarding spatial localisation within the brain was limited. Here we applied MSI
 284 to study the metabolic processes along the MGB axis, permitting imaging of multiple
 285 neurotransmitters in the presence and absence of a microbiome while untargeted MSI also
 286 allowed the discovery of novel metabolites involved in MGB axis communication.

287 Serotonin, tryptophan, dopamine, tyrosine, 3-methoxytyramine, GABA and glutamate were
 288 first imaged to detect changes across the brain and gut of GF animals. No changes were
 289 detected in the brain whilst in the intestine only tyrosine and glutamate were increased and
 290 serotonin decreased in GF animals compared to SPF controls. Significantly lower levels of
 291 serotonin have previously been detected in the colon of GF mice along with increased levels
 292 of the serotonin precursor tryptophan (32). Whilst there was a trend towards increased levels
 293 of tryptophan in the present study this was not significant. No difference in serotonin or
 294 tryptophan levels were observed across the brain sections of GF mice in comparison to SPF
 295 animals here, in contrast to previous reported GF studies (14, 47). However, both rat and
 296 mouse models have been used in these studies, complicating interpretation of data that has
 297 also indicated that these differences can be sex-specific. Whilst microbiome changes within
 298 mouse colonies as well as between mouse strains are well documented, phenotypic
 299 comparisons between GF rodents which lack a microbiome have been limited (48–50).

300 One week antibiotic (ABX) treated mice were also tested for changes in levels of serotonin
 301 and tryptophan in the gut and brain compared to untreated controls. Tryptophan abundance
 302 increased in both the colon and the brain of ABX treated mice compared to untreated
 303 controls, but serotonin levels remained unchanged. This data mirrors that previously obtained
 304 with a similar ABX model, where ABX treatment increased systemic tryptophan whilst
 305 serotonin levels remained unaffected (19, 34).

306 Dopamine and its precursor tyrosine were also affected by microbiota disruption or absence.
307 Increased tyrosine and dopamine levels were detected in the colon in ABX treated mice while
308 GF mice had increased intestinal tyrosine levels compared to controls. No difference was
309 found in the abundance of either of these molecules in brains from either ABX treated or GF
310 mice when compared to controls. Previous work found a decrease in the level of dopamine in
311 the guts of GF mice, whilst another found no difference in the levels of dopamine in the colon
312 after ABX treatment, although antibiotic treatments varied by constitution and duration
313 between ours and previous studies (28)(16). As tyrosine can be metabolized by certain
314 bacterial species, it is possible that reduction in these groups could lead to higher production
315 of dopamine (51). Conversely the release of a biologically active, free form of dopamine in the
316 gut via bacterial β -glucuronidase mediated breakdown of a conjugated form of dopamine
317 could also play a significant role (28). Additionally, although there are no differences in the
318 levels of dopamine in the brain, increased levels in the colon could have localised effects.
319 There are dopamine receptors present in the intestine and dopamine, in a similar manner to
320 serotonin, has been shown to increase water absorption from the gut and regulation of
321 muscle contraction (28, 52, 53).

322 The neurotransmitter, and GABA precursor, glutamate was increased in the colon of GF mice
323 compared to controls. Glutamate and GABA are the main excitatory and inhibitory
324 neurotransmitters of the central nervous system, respectively (54). The increase in glutamate
325 levels in GF mice was surprising given the number of bacteria known to produce glutamate in
326 the intestine (55). This build-up of glutamate is not due to reduced conversion to GABA as we
327 detected no corresponding difference in GABA levels (56). No increase in glutamate in the
328 GF brain was detected in these GF mice with high intestinal glutamate levels, but glutamate
329 is primarily metabolised in the splanchnic area and little enters into circulation from the
330 gastrointestinal tract, instead exerting its significant localised effects on the gut including
331 through stimulation of the vagus nerve (57, 58).

332 Untargeted MSI indicated that three metabolites were significantly altered in the brain, vitamin
 333 B5 or pantothenic acid (m/z 218.1030) and 3-hydroxy-3-methylglutaric acid (3-HMG)(m/z
 334 161.0446) and m/z 160.133 (Hulme et al., In press). The latter m/z was determined to be in
 335 fact a mixture of two molecules which are discussed in detail elsewhere (Hulme *et al.*, In
 336 press). Vitamin B5 was significantly decreased in GF mice compared to SPF, whereas 3-
 337 HMG was increased. Further analysis of vitamin B5 and HMG determined that they were not
 338 significantly altered in ABX treated mice. Vitamin B5 is a precursor for co-enzyme A, which is
 339 an important molecule in many metabolic pathways including neurotransmitter production.
 340 Previously it was thought that only small amounts of vitamin B5 could be produced by the
 341 microbiota but recent work identified a previously uncharacterised group of *Clostridia* that
 342 harbour genes for pantothenic acid biosynthesis (59, 60). Vitamin B5 is involved in oxidative
 343 metabolism and the synthesis of neurotransmitters including acetylcholine (61). Neurological
 344 symptoms of vitamin B5 deficiency were determined in early studies by inducing deficiency in
 345 human subjects, which resulted in defects in neuromuscular function and deterioration of
 346 mood (62). It has also recently been determined that levels of vitamin B5 producing bacteria
 347 change according to gestational age in preterm infants (60). The lower levels in the GF brain
 348 were particularly apparent in the cerebellum, hippocampus and hypothalamus brain regions.
 349 Despite the potential for a microbial origin for vitamin B5, no change was found in the levels
 350 of vitamin B5 in the colons of GF mice making it unclear what role, if any, the microbiota may
 351 play in the significant decrease in vitamin B5 levels in the brain. While vitamin B5 crosses into
 352 the brain through the BBB via a saturable transporter and levels are maintained in the brain at
 353 around 50 times the concentration found in the plasma, the mechanism of efflux of this
 354 vitamin from the brain is unclear and it may also be possible that increased BBB permeability
 355 in GF mice could lead to increased efflux from the brain (63, 64)(10).
 356 HMG, a metabolite involved in leucine degradation and ketogenesis was present at higher
 357 levels across the brains of GF mice compared to normal brains, but particularly in the
 358 cerebellum. Individuals with the genetic disorder 3-hydroxy-3-methylglutaric aciduria, which

359 leads to a build-up of metabolites including HMG, suffer from neurological symptoms
 360 including seizures and abnormalities in the brain (65). It has been proposed that this
 361 accumulation of metabolites is directly affecting the brain with HMG inducing oxidative
 362 damage (65–68). Presence of HMG in the serum has been associated with increased gut
 363 permeability in children with environmental enteric dysfunction (69). Studies have shown that
 364 bacteria play a role in maintaining intestinal barrier function, therefore, the increased intestinal
 365 permeability seen in GF guts could be leading to higher levels of HMG in circulation
 366 compared to normal mice (70). Furthermore, intraperitoneal injections of HMG into rats leads
 367 to high levels accumulating in the brain of 7 day old rats but not in 30 day old rats in what was
 368 speculated to be a BBB permeability related effect (65). Therefore given the known BBB, and
 369 likely intestinal, permeability defects in GF mice, HMG, and potentially other metabolites, are
 370 likely entering the circulation and subsequently the brain at higher levels (10). Furthermore,
 371 no significant difference was found in the levels of HMG in the brain of ABX treated mice.

372

373 **Conclusions:**

374 This study has highlighted the capabilities and potential of MSI to enhance investigation of
 375 the MGB axis through the detection and discovery of molecules involved in MGB
 376 communication. Here we show that, despite significant changes in gut microbiota,
 377 neurotransmitters are not significantly changed in the brain. As the significance of the MGB
 378 axis is still being realised, MSI offers a unique opportunity to understand the complexity of
 379 these interactions by identifying both the known and unknown mediators of host-microbe
 380 communication.

381 **Declarations**

382 **Ethics approval and consent to participate**

383 Approval for animal procedures was given prior to their initiation by internal University of
384 Manchester and University of Glasgow ethics committees and by the U.K. Home Office under
385 licenses 70/7815, PPL40/4500, P64BCA712 and P78DD6240.

386

387 **Consent for publication**

388 Not applicable

389

390 **Availability of data and material**

391 The datasets during and/or analysed during the current study available from the
392 corresponding author on reasonable request.

393

394 **Competing interests**

395 The authors declare that they have no competing interests.

396

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402

403 **Authors' contributions**

404 H.H., L.M.M., R.J.A.G., R.B. and D.M.W. planned the studies. H.H., L.M.M., N.S., J.S., G.H.
405 R.A.B., S.L.B., A.S.M., and D.M.W. conducted experiments. H.H., L.M.M., N.S., J.S., G.H.,
406 S.M., R.J.A.G., R.B. and D.M.W interpreted the studies. H.H., L.M.M., R.J.A.G., R.B. and

407 D.M.W. wrote the first draft of the paper. All authors reviewed, edited and approved the
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409

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411

412

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659 **Figure Titles and Legends**

660 **Figure 1. Effects of the gut microbiome on neurotransmitters, neurotransmitter** 661 **precursors and neurotransmitter metabolites in the murine brain and gut.**

662 DESI-MS images of various DPP-derivatised neurotransmitters, neurotransmitter precursors
663 or neurotransmitter metabolites in: (a) germ-free (GF) and specific-pathogen-free (SPF)
664 mouse brains (N=5), (b) GF and SPF mice colons (N=4), (c) antibiotic treated (ABX) and
665 untreated (Unt) mouse brains (N=5), (d) and ABX and Unt mice colons (N=5). H and E
666 stained sections are shown, which are the same tissue sections that underwent DESI-MSI
667 analysis. The bar plots show the average metabolite abundance (ion intensity) across the
668 whole tissue sections in the different groups tested. For (b), statistical analysis was performed
669 using an unpaired t test. For (c) and (d), statistical analysis was performed using a paired t
670 test, an unpaired t test or a Mann-Whitney U test where appropriate. Error bars represent
671 standard deviation. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Scale bars = 2 mm.

672

673 **Figure 2. Impact of the gut microbiome on 3-hydroxy-3-methylglutaric acid levels in the**
674 **gut and brain.** DESI-MS images of HMG in the (a) GF and SPF mouse brains (N=5), (b) GF
675 and SPF mice colons (N=5), (c) ABX treated and Unt mouse brain (N=3), (d) and ABX treated
676 and Unt colons (N=3). H and E stained sections are shown, which are the same tissue

677 sections that underwent DESI-MSI analysis. The bar plots show the average HMG
678 abundance (ion intensity) across the whole tissue sections in the different groups tested.
679 Annotation in (a) Cb, cerebellum. Statistical analysis was performed using an unpaired t test.
680 Error bars represent standard deviation N=5. ****, $p \leq 0.0001$. Scale bars=2 mm.

681

682 **Figure 3. Effects of the gut microbiome on pantothenic acid (B5) levels in the gut and**
683 **brain.** DESI-MS images of B5 in the (a) GF and SPF mouse brains (N=5), (b) GF and SPF
684 mice colons (N=5), (c) ABX treated and Unt mouse brain (N=3), (d) and ABX treated and Unt
685 colons (N=3). H and E stained sections are shown, which are the same tissue sections that
686 underwent DESI-MSI analysis. The bar plots show the average B5 abundance (ion intensity)
687 across the whole tissue sections in the different groups tested. Annotation in (a) Cb,
688 cerebellum; Hip, hippocampus; Hy, hypothalamus. Statistical analysis was performed using a
689 paired t test. Error bars represent standard deviation N=5. *, $p \leq 0.05$. Scale bars=2 mm.

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704 **Figures and Tables**

705 **Table 1 DESI-MSI parameters**

Molecule	Pump	Spatial	Ionisation	Mass	S-lens	Mass	Injection
	solvent	resolution	mode	range	setting	resolution	time
Neurotransmitters	1.5	100 μ m	Positive	200-	75	35000	150
	μ l/min			800			
Pantothenic acid	1.5	100 μ m	Negative	65-	50	70000	300
	μ l/min			400			
3-hydroxy-3-	1.5	100 μ m	Positive	65-	50	70000	300
methylglutaric	μ l/min			400			

706

707

708 **Figure 1**

718 Figure 2

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720

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722

723 Figure 3

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728 SUPPLEMENTARY INFORMATION

729 Supplementary methods

730 *MALDI-MSI analysis*

731 For quantification analysis of HMG, MALDI-MSI analysis was performed using the following
 732 parameters: α -Cyano-4-hydroxycinnamic acid (CHCA) matrix was applied at 5 mg/mL
 733 concentration in 50% acetonitrile, 50% water with 0.1% trifluoroacetic acid using an automated
 734 matrix applicator (HTX technologies, Chapel Hill, NC, US); 8 passes were performed using a
 735 nozzle temperature of 75°C, velocity of 1100 mm/min, flow rate of 80 μ L/min, and gas
 736 pressure of 6 psi. 9-aminoacridine matrix was used at a concentration of 10 mg/mL in 80%
 737 methanol 20% water and sonicated for 20 min. The matrix was applied using an automated
 738 matrix applicator (HTX technologies, Chapel Hill, NC, US) for 3 passes, using a nozzle
 739 temperature of 75°C, gas pressure of 6 psi, velocity of 1120 mm/min and flow rate of 80
 740 μ L/min.

741 MALDI-MSI was carried out on a Rapiflex MALDI-TOF instrument (Bruker Daltonics, Bremen,
 742 Germany) with a 10 kHz smartbeam laser. A spatial resolution of 50 μ m was achieved using
 743 a single 5 μ m laser beam mode, with 600 laser shot over an area of 50x50 μ m. Results were
 744 analysed using Fleximaging v5 software and the data was normalized to total ion count.

745

746 *3-hydroxy-3-methylglutaric acid (HMG) and pantothenic acid (vitamin B5) quantitation*

747 Labelled standards for 3-hydroxy-3-methyl-d3-pentanedioic acid and vitamin B5 (*di- β -alanine-*
 748 $^{13}\text{C}_6$, $^{15}\text{N}_2$) calcium salt (Sigma-Aldrich, Poole, Dorset, UK) were spotted onto a brain section
 749 using a Preddator reagent dispenser (Redd & Whyte, London, UK). Brain sections with the
 750 spotted standards were imaged alongside the experimental brain sections from SPF and GF
 751 mice using DESI-MSI for vitamin B5 and MALDI-MSI for HMG. The standards were then used
 752 to generate standard curves for mean intensity against concentration for each standard spot.
 753 These values were used to calculate the concentration of the endogenous molecule across

the experimental brain sections considering the thickness and area of tissue section analysed.

756

757 **Supplementary figures**

758 **Figure S1.**

759

760 **Effects of the gut microbiome on neurotransmitters, neurotransmitter**

761 **precursors and neurotransmitter metabolites in the murine brain and gut. Bar**

762 plots showing the average metabolite abundances in the brain and colon of germ-free

763 mice (GF) compared to specific-pathogen-free mice (SPF). Error bars represent

764 standard deviation. Brain N=5, colons N=4.

765

766

Figure S2

Effects of the gut microbiome on neurotransmitters, neurotransmitter precursors and neurotransmitter metabolites in the murine brain and gut. Bar plots showing the average metabolite abundances in the brain and colon of one week antibiotic treated mice (ABX) compared to untreated control mice (Unt). Error bars represent standard deviation. N=5.

776 Figure S3

777

778 **Identification of metabolite at m/z 161.0446.** Product ion spectra from MS/MS

779 analysis of the metabolite at m/z 161.0446 compared to product ion spectra from

780 MS/MS analysis of 3-hydroxy-3-methylglutaric acid standard. The spectra show the

781 m/z range across the x-axis and arbitrary unit on the y-axis. The MS/MS analysis was

782 performed at different high-energy collision dissociation voltages (HCD 15-100).

783

784 **Figure S4**

785

786 **Absolute quantification of HMG in GF and SPF brain.** (a) MALDI-MSI ion image of
787 HMG (m/z 161.044) in the GF brain, which has been spotted with various
788 concentrations of deuterated HMG standard, shown in (b). (c) Overlay of endogenous
789 HMG and deuterated HMG standard. (d) Calibration curve obtained from the various
790 concentrations of deuterated HMG standard spotted on the brain section. (e-g)

791 Comparison of the HMG MALDI-MSI ion images in the GF and SPF mouse brain and
792 absolute quantification values across the whole brain and the cerebellum.

793 **Figure S5**

794

795 **Identification of the metabolite at m/z 218.102.** Product ion spectra from MS/MS

796 analysis of the metabolite at m/z 218.102 compared to product ion spectra from

797 MS/MS analysis of vitamin B5 standard. The spectra show the m/z range across the

798 x-axis and arbitrary unit on the y-axis. The MS/MS analysis was performed at different

799 high-energy collision dissociation voltages (HCD 15-65).

800

801 **Figure S6**

802

803 **Absolute quantification of vitamin B5 in GF and SPF brain.** (a) DESI-MSI ion

804 image of B5 (m/z 218.103) in the GF brain, which has been spotted with various

805 concentrations of ^{13}C labelled B5 standard, shown in (b). (c) Overlay of endogenous

806 HMG and ^{13}C labelled B5 standard. (d) Calibration curve obtained from the various

807 concentrations of deuterated ^{13}C labelled B5 standard spotted on the brain section.

808 (e-g) Comparison of the B5 DESI-MSI ion images in the GF and SPF mouse brain

809 and absolute quantification values across the whole brain and the cerebellum.

810