

1 **Regulation of blood–brain barrier integrity and cognition by the microbiome-**  
2 **associated methylamines trimethylamine-*N*-oxide and trimethylamine**

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28

29 **Running title:** Dietary methylamines affect the blood–brain barrier

30 **Abbreviations:** BBB, blood–brain barrier; DI, discrimination index; GO, gene  
31 ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS,  
32 lipopolysaccharide; NOR, novel object recognition; SCFA, short-chain fatty acid; OFT,  
33 open field test; SPIA, signalling pathway impact analysis; TEER, transendothelial  
34 electrical resistance; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide.

35

36 Supplementary materials associated with the article are available from figshare

37 (<https://doi.org/10.6084/m9.figshare.13549334.v1>).

38 **ABSTRACT**

39 Communication between the gut microbiota and the brain is primarily mediated *via*  
40 soluble microbe-derived metabolites, but the details of this pathway remain poorly  
41 defined. Methylamines produced by microbial metabolism of dietary choline and L-  
42 carnitine have received attention due to their proposed association with vascular  
43 disease, but their effects upon the cerebrovascular circulation have not hitherto been  
44 studied. Here we use an integrated *in vitro/in vivo* approach to show that  
45 physiologically relevant concentrations of the dietary methylamine trimethylamine *N*-  
46 oxide (TMAO) enhanced and protected blood-brain barrier (BBB) integrity, acting  
47 through the tight junction regulator annexin A1. In contrast, the TMAO precursor  
48 trimethylamine (TMA) impaired BBB function and disrupted tight junction integrity.  
49 Moreover, we show that long-term exposure to TMAO has beneficial effects upon  
50 cognition in mice, improving visual recognition memory. Our findings demonstrate a  
51 direct interaction of microbiome-associated metabolites with the mammalian BBB, with  
52 consequences for cerebrovascular and cognitive function.

53

54

## 55 INTRODUCTION

56 As the role of the gut microbiota in host physiology and disease is categorised, novel  
57 pathways through which these interactions are mediated continue to emerge. We and  
58 others recently identified the blood–brain barrier (BBB) as a target for gut microbe-  
59 derived short-chain fatty acid (SCFA) activity, with butyrate and propionate acting to  
60 promote BBB integrity and protect the cerebral vasculature from insult<sup>1,2</sup>. SCFAs  
61 represent just one of many classes of gut microbe-derived metabolites, with little  
62 known as to how these other classes may influence BBB function.

63  
64 Dietary methylamines, such as choline, phosphatidylcholine, betaine and  
65 trimethylamine-*N*-oxide (TMAO), are a class of metabolites receiving considerable  
66 attention as modulators of vascular function<sup>3,4</sup>, although the mechanism(s) by which  
67 they affect human physiology remain poorly understood. The aforementioned  
68 methylamines can be broken down by members of the gut microbiota into  
69 trimethylamine (TMA)<sup>5</sup>, which is carried from the gut through the portal vasculature to  
70 the liver and rapidly converted into TMAO by flavin monooxygenases<sup>6</sup>. TMAO then  
71 enters the systemic circulation, reaching fasting plasma concentrations of between 2  
72 and 40  $\mu$ M in humans<sup>7–9</sup>, prior to excretion through the urine<sup>5</sup>. Approximately ten-fold  
73 lower concentrations of TMA compared with TMAO are found in the circulation under  
74 normal physiological conditions.

75  
76 Early observational work reported an apparent association between atherosclerosis  
77 and elevated levels of TMAO<sup>10,11</sup>. Similarly, pre-clinical studies demonstrate the  
78 damaging effects of supraphysiological TMAO doses in atherosclerosis-prone mice<sup>12</sup>  
79 and upon thrombus formation<sup>13</sup>. Despite this, the impact of TMAO upon the  
80 vasculature remains uncertain, with a number of detailed studies encompassing both  
81 human and murine systems failing to replicate these initial findings<sup>14</sup>, instead  
82 suggesting that this negative relationship disappears upon correction for renal  
83 function<sup>4,15–17</sup>. This indicates that the raised TMAO levels may in fact reflect impaired  
84 excretion rather than being a causative factor of disease. Moreover, protective roles  
85 for TMAO have been reported in rodent models of hypertension<sup>18</sup>, atherosclerosis<sup>19</sup>  
86 and non-alcoholic steatohepatitis<sup>20</sup> and we have previously shown TMAO to improve  
87 glucose homeostasis and insulin secretion in mice fed a high-fat diet<sup>21</sup>. Perhaps  
88 helping to clarify this apparent contradiction, recent studies have established that

89 intravenous treatment of rats with the TMAO precursor TMA, but not TMAO itself,  
90 increases mean arterial blood pressure<sup>22</sup>. Notably, the majority of reports describing  
91 associations of plasma TMAO with cardiovascular disease have not concurrently  
92 monitored levels of TMA; TMA but not TMAO has been shown to associate with severe  
93 aortic stenosis<sup>22</sup> and gestational diabetes risk<sup>23</sup>.

94

95 Beyond vascular health, dietary methylamines have implications for cognition, with a  
96 positive correlation observed between choline intake and cognitive function in both  
97 humans<sup>24,25</sup> and mice<sup>26,27</sup>. In contrast, cerebrospinal fluid TMAO levels have been  
98 indicated as predictive of cognitive decline in Alzheimer's disease<sup>28</sup>, while suppression  
99 of microbial TMA/TMAO production improves cognitive function in the murine  
100 APP/PS1 model of Alzheimer's disease<sup>29</sup>. Given the disparities in the literature  
101 regarding the effects of methylamines upon the vasculature, and our increasing  
102 awareness of the BBB as a major actor in the pathology of multiple neurological  
103 conditions, we investigated the effects of physiologically relevant concentrations of  
104 TMAO and its precursor TMA upon BBB integrity and cognitive behaviour.

105

## 106 **METHODS**

107

### 108 *Endothelial cell culture*

109 The human cerebromicrovascular endothelial cell line hCMEC/D3 was maintained and  
110 treated as described previously<sup>2,30</sup>. Cells bearing shRNA sequences targeting annexin  
111 A1 (*ANXA1*) or non-specific scramble sequences were produced as described  
112 previously<sup>31</sup>; the degree of *ANXA1* knock-down was confirmed by flow cytometry  
113 analysis (Suppl. Fig. 1). For all lines, cells were cultured to confluency in complete  
114 EBM-2MV microvascular endothelial cell growth medium (Promocell GmbH,  
115 Heidelberg, Germany), whereupon medium was replaced by EBM-2MV without VEGF  
116 and cells were further cultured for a minimum of 4 days to enable intercellular tight  
117 junction formation prior to experimentation.

118

### 119 *In vitro barrier function assessments*

120 Paracellular permeability and transendothelial electrical resistance (TEER) were  
121 measured on 100 % confluent hCMEC/D3 cultures polarised by growth on 24-well  
122 plate polyethylene terephthalate (PET) transwell inserts (surface area: 0.33 cm<sup>2</sup>, pore  
123 size: 0.4 µm; Greiner Bio-One GmbH, Kremsmünster, Austria) coated with calf-skin  
124 collagen and fibronectin (Sigma-Aldrich, UK). The permeability of hCMEC/D3 cell  
125 monolayers to 70 kDa FITC-dextran (2 mg/ml) was measured as described  
126 previously<sup>31-33</sup>. TEER measurements were performed using a Millicell ERS-2  
127 Voltohmmeter (Millipore, Watford, UK) and were expressed as Ω.cm<sup>2</sup>. In all cases,  
128 values obtained from cell-free inserts similarly coated with collagen and fibronectin  
129 were subtracted from the total values. In some cases, barrier integrity was tested by  
130 challenge with bacterial lipopolysaccharide (LPS). Confluent hCMEC/D3 monolayers  
131 were treated with TMAO or TMA for 12 h, whereupon LPS (*Escherichia coli* O111:B4;  
132 50 ng/ml, comparable to circulating levels of LPS in human endotoxemia<sup>34</sup>) was added  
133 for a further 12 h, without wash-out. Barrier function characteristics were then  
134 interrogated as described above.

135

### 136 *Cell adhesion assays*

137 hCMEC/D3 cells were cultured to confluency on transwell inserts (0.4 µm pore size,  
138 0.33 cm<sup>2</sup> diameter, Greiner Bio-One GmbH, Austria) prior to 16 h treatment with 10  
139 ng/ml TNFα. Monolayers were then incubated for 2 h with U937 monocytic cells pre-

140 labelled according to manufacturer's instructions with CMFDA cell tracker dye  
141 (ThermoFisher Scientific, UK). Co-cultures were washed vigorously with ice-cold PBS  
142 three times and fixed by incubation for 10 min in 1 % formaldehyde in 0.1 M PBS. Co-  
143 cultures were mounted and examined using an Axiovert 200M inverted microscope  
144 (Zeiss) equipped with a 20x objective lens. Images were captured with ZEN imaging  
145 software (Carl Zeiss Ltd, UK) and analysed using ImageJ 1.53c (National Institutes of  
146 Health, USA).

147

#### 148 *Microarrays*

149 hCMEC/D3 cells were grown on 6-well plates coated with calf-skin collagen (Sigma-  
150 Aldrich, Gillingham, UK), and collected in TRIzol (Thermo-Fisher Scientific, UK) as  
151 described previously<sup>2</sup>. Total RNA was extracted using a TRIzol Plus RNA purification  
152 kit (Thermo-Fisher Scientific, UK) and quantified using a CLARIOstar  
153 spectrophotometer equipped with an LVis microplate (BMG Labtech GmbH,  
154 Germany).

155

156 Hybridization experiments were performed by MacroGen Inc. (Seoul, Republic of  
157 Korea) using Illumina HumanHT-12 v4.0 Expression BeadChips (Illumina Inc., San  
158 Diego, CA) as described previously<sup>2</sup>.

159

#### 160 *Processing and analyses of array data*

161 Raw data supplied by MacroGen were quality-checked, log<sub>2</sub>-transformed and loess-  
162 normalized (2 iterations) using affy<sup>35</sup>. Probe filtering and matching of probes not  
163 classified as 'Bad' or 'No match' to Entrez identifiers were done as described  
164 previously<sup>2</sup>. Average gene expression values were used for identification of  
165 differentially expressed genes. Array data have been deposited in ArrayExpress under  
166 accession number E-MTAB-6662. Normalized data are available (Supplementary  
167 Table 1).

168

169 Enrichr<sup>36,37</sup> was used to perform Gene Ontology (GO) analysis. Signaling Pathway  
170 Impact Analysis (SPIA) was used to determine whether Kyoto Encyclopedia of Genes  
171 and Genomes (KEGG) pathways were activated or inhibited in hCMEC/D3 cells  
172 exposed to TMAO or TMA<sup>38</sup>. Human KEGG pathways (KGML format) downloaded

173 from the KEGG PATHWAY database<sup>39</sup> were used for network (KEGGgraph, RBGL<sup>40</sup>)  
174 analysis.

175

#### 176 *Immunofluorescence microscopy*

177 hCMEC/D3 cells were cultured on 24-well plate polyethylene terephthalate (PET)  
178 transwell inserts (surface area: 0.33 cm<sup>2</sup>, pore size: 0.4 µm; Greiner Bio-One GmbH,  
179 Kremsmünster, Austria) coated with calf-skin collagen and fibronectin (Sigma-Aldrich,  
180 UK), prior to immunostaining according to standard protocols<sup>2,31</sup> and using a primary  
181 antibody directed against zonula occludens-1 (ZO-1; 1:100, ThermoFisher Scientific,  
182 UK) or Alexafluor 488-conjugated phalloidin (1:140, ThermoFisher Scientific, UK).  
183 Nuclei were counterstained with DAPI (Sigma-Aldrich, UK). Images were captured  
184 using an LSM880 confocal laser scanning microscope (Carl Zeiss Ltd, Cambridge,  
185 UK) fitted with 405 nm and 488 nm lasers, and a 63x oil immersion objective lens (NA,  
186 1.4 mm, working distance, 0.17 mm). Images were captured with ZEN imaging  
187 software (Carl Zeiss Ltd, UK) and analysed using ImageJ 1.53c (National Institutes of  
188 Health, USA).

189

#### 190 *Flow cytometry analysis*

191 Following experimental treatment, hCMEC/D3 cells were detached using 0.05%  
192 trypsin and incubated with an unconjugated rabbit polyclonal antibody directed against  
193 ANXA1 (1:1000, ThermoFisher Scientific, UK) on ice for 30 minutes, followed by  
194 incubation with an AF488-conjugated goat anti-rabbit secondary antibody (1:500,  
195 ThermoFisher Scientific, UK). Similarly detached hCMEC/D3 cells were incubated  
196 with APC-conjugated mouse monoclonal anti-BCRP (1:100, BD Biosciences, Oxford,  
197 UK), or PE-conjugated mouse monoclonal anti-MDR1A (1:100, BD Biosciences, UK)  
198 antibodies on ice for 30 minutes, alongside fluorescence minus one controls.  
199 Immunofluorescence was analysed for 20,000 events per treatment using a BD  
200 FACSCanto II (BD Biosciences, UK) flow cytometer; data were analysed using FlowJo  
201 8.0 software (Treestar Inc., CA, USA).

202

#### 203 *Efflux transporter assays*

204 Activity of the major efflux transporters P-glycoprotein and BCRP was determined  
205 through the use of commercially available assays (PREDEASY™ ATPase Assay Kits,  
206 Solvo Biotechnology Inc., Budapest, Hungary), performed according to the



207 manufacturer's instructions. Stepwise dose–response curves centred around reported  
208 physiological circulating concentrations of TMA (4.9 nM – 10.8  $\mu$ M) and TMAO (0.5  
209  $\mu$ M – 1.08 mM) were constructed ( $n=4$ ) to investigate inhibitory effects of the  
210 methylamines upon transporter activity.

211

## 212 *ELISA*

213 Culture medium ANXA1 content was assayed by specific ELISA as described  
214 previously<sup>41</sup>. Serum TNF $\alpha$  and IL-1 $\beta$  concentrations were assayed using commercial  
215 ELISA kits according to the manufacturer's instructions (ThermoFisher Scientific, UK).

216

## 217 *Animal experiments*

218 All animal experiments were performed according to the UK Animals (Scientific  
219 Procedures) Act of 1986, under UK Home Office Project Licences PFA5C4F4F (short  
220 term studies) and 70/8710 (long term studies), following ethical review by the Animal  
221 Welfare and Ethical Review Boards of Queen Mary, University of London or the  
222 University of East Anglia, respectively. Wild-type male C57Bl/6J mice (Charles River  
223 Ltd., Harlow, UK) aged 8 weeks at the start of procedures were used throughout, with  
224 a group size of  $n=6$  for short term studies and  $n=8$  for long-term/behavioural analyses.  
225 Animals were housed in individually ventilated cages on a daily 12 h:12 h light/dark  
226 cycle with, unless otherwise indicated, *ad libitum* access to standard mouse chow and  
227 drinking water. Experimental procedures were started at 9 am to minimise variation  
228 associated with circadian rhythms.

229

## 230 *Assessment of acute effects of TMAO on BBB integrity*

231 Mice ( $n=4-6$  per group) were injected i.p. with 1.8 mg/kg body weight TMAO in 100  $\mu$ l  
232 saline vehicle, a dose calculated to approximate human circulating TMAO levels<sup>42</sup>,  
233 followed 2 h, 6 h or 24 h later by assessment of Evans blue extravasation as described  
234 below. Alternatively, mice were injected i.p. with 3 mg/kg body weight LPS or 100  $\mu$ l  
235 0.9% saline vehicle, followed 2 h later by i.p. injection of either 1.8 mg/kg body weight  
236 TMAO or 100  $\mu$ l 0.9% saline vehicle for assessment of Evans blue extravasation 2 h  
237 later. In both experiments, one hour before assessment animals were injected i.p. with  
238 100  $\mu$ l of a 2% (w/v) solution of Evans blue dye in 0.9 % saline (Sigma–Aldrich Ltd,  
239 Poole, UK). Dye was permitted to circulate for 1 h before animals were transcardially

240 perfused with 0.9% saline at 4 °C to remove circulating dye. Brains were removed,  
241 bisected and homogenized by maceration in 0.9% saline. Suspended macromolecules  
242 were precipitated by incubation with 60% trichloroacetic acid, and dye content of  
243 resulting supernatants was detected using a CLARIOstar spectrophotometer (BMG  
244 Labtech GmbH, Germany) alongside a standard curve of defined concentrations of  
245 Evans blue in the same buffer. Brain Evan's blue content was expressed as µg of dye  
246 per mg of brain tissue, normalized to circulating plasma concentrations.

247

#### 248 *Long-term LPS and TMAO treatments*

249 To assess the long-term impact of both LPS and TMAO on cognitive performance,  
250 mice were divided into four groups (n=8 per group): 1) Water + PBS; 2) Water + TMAO;  
251 3) LPS + PBS; 4) LPS + TMAO. C57Bl/6 mice were administered phosphate-buffered  
252 saline (PBS) or LPS (*Escherichia coli* O55:B5, Sigma-Aldrich, UK) via intraperitoneal  
253 (i.p.) injection (0.5 mg/kg/wk) for 8 weeks<sup>43</sup>. A final LPS treatment was administered  
254 the day before sacrifice for nine total injections. Body weights were recorded prior to  
255 each injection. TMAO was provided in the drinking water (500 mg/L) and water bottles  
256 replaced every other day. Drinking volumes were recorded before bottle change.

257

#### 258 *Processing and analyses of RNAseq data*

259 Mice were transcardially perfused with 0.9% saline at 4 °C to remove circulating blood,  
260 and brains were removed and collected into RNAlater (ThermoFisher Scientific Ltd.,  
261 UK) prior to storage at -20 °C for later analysis. Whole brain total RNA was extracted  
262 using a PureLink RNA Mini Kit (ThermoFisher Scientific Ltd., UK) and quantified using  
263 a CLARIOstar spectrophotometer equipped with an LVis microplate (BMG Labtech  
264 GmbH, Germany). RNA samples (n=3 TMAO, n=3 control) were sent to Macrogen Inc.  
265 (Republic of Korea) where they were subject to quality checks (RIN analysis); libraries  
266 were prepared (TruSeq Stranded mRNA LT Sample Prep Kit) for paired-end (2x 100  
267 nt) sequencing on an Illumina HiSeq 4000 apparatus. Raw RNAseq sequence data  
268 (delivered in fastq format) were processed in house as follows. Reads were mapped  
269 onto the mouse genome (mm10) using HISAT2 v2.1.0<sup>44</sup>. Number of reads in each  
270 sample that mapped to genes in the BAM files returned by HISAT2 was determined  
271 using featureCounts v1.6.4<sup>45</sup>. Entrez gene identifiers were converted to gene symbols  
272 using *Mus musculus* annotations downloaded from NCBI on 26 November 2020; only  
273 those genes with valid Entrez gene identifiers were retained in analyses. Raw RNAseq

274 data have been deposited with ArrayExpress under accession number E-MTAB-9869.  
275 Significantly differentially expressed genes ( $P < 0.1$ ) were analysed by mouse KEGG  
276 pathway over-representation analysis using Enrichr and manual curation.

277

### 278 *Behavioural analyses*

279 Behavioural tests were performed in the order they are introduced below. Apparatus  
280 was cleaned using 70 % ethanol upon completion of each trial, eliminating any residual  
281 odour.

282

283 Open field test (OFT) was conducted as previously described<sup>46</sup>. Briefly, mice were  
284 placed in the centre of the OFT, a grey 50 x 50 x 50 cm apparatus illuminated with low  
285 lux (100 lux) lighting. Total travel distance and time spent in the centre of the field was  
286 determined at 5 min with a video tracking system (Smart 3.0 tracking software, Panlab,  
287 Kent, UK).

288

289 The novel object recognition (NOR), a measure of recognition memory, was performed  
290 as described previously<sup>47,48</sup>, with slight modifications. Briefly, on day 1 mice were  
291 habituated in a grey 50 x 50 x 50 cm apparatus illuminated with low lux (100 lux)  
292 lighting, mice were placed into the empty maze and allowed to move freely for 10 min.  
293 On day 2, mice were conditioned to a single object for a 10 min period. On day 3, mice  
294 were placed into the same experimental area in the presence of two identical objects  
295 for 15 min, after which they were returned to their respective cages and an inter-trial  
296 interval of 1 h was observed. One familiar object was replaced with a novel object.  
297 Mice were placed back within the testing area for a final 10 min. Videos were analysed  
298 for a 5 min period, after which if an accumulative total of 15 s with both objects failed  
299 to be reached, analysis continued for the full 10 min or until 15 s was achieved. Those  
300 not achieving 15 s were excluded from the analysis<sup>49</sup>. A discrimination index (DI) was  
301 calculated as follows:  $DI = (TN - TF) / (TN + TF)$ , where TN is the time spent exploring  
302 the novel object and TF is the time spent exploring the familiar object.

303

304 Y-maze spontaneous alternation test, a measure of spatial working memory, was  
305 performed on the final day of behavioural testing as previously described<sup>50</sup>. Briefly, the  
306 Y-maze apparatus comprised white Plexiglas (dimensions 38.5 x 8 x 13 cm, spaced  
307 120° apart) and was illuminated with low lux (100 lux) lighting. Mice were placed in the

308 maze and allowed to explore freely for 7 min while tracking software recorded zone  
309 transitioning and locomotor activity (Smart 3.0 tracking software, Panlab, Kent, UK).  
310 Spontaneous alternation was calculated using the following formula: Spontaneous  
311 Alternation = (Number of alternations/ Total Arm entries - 2) x 100.

312

### 313 *Extravasation assay and sample processing following long-term treatment*

314 Twenty-four hours after the final injection of LPS, mice were injected i.p. with 200 µl of  
315 2% sodium fluorescein in sterile ddH<sub>2</sub>O and anesthetized 30 min later with isoflurane  
316 (1.5%) in a mixture of nitrous oxide (70%), and oxygen (30%). Once sedated, blood  
317 was collected by cardiac puncture and centrifuged at 1,500 × g for 15 min at 4 °C to  
318 collect the serum. The samples were analysed immediately for sodium fluorescein  
319 extravasation or snap-frozen in liquid nitrogen and stored at -80 °C until further  
320 analysis.

321

322 Mice were then transcardially perfused with saline containing 10 kU/ml heparin  
323 (Sigma, Devon, UK). Dissected left hemi-brains were fixed in 4% PFA for 24 h and  
324 embedded into paraffin before being processed for immunohistochemical analysis.  
325 Right hemi-brains were stored at -80 °C until further analysis; cerebellums were  
326 processed immediately for the sodium fluorescein extravasation assay. Cleared  
327 volume of sodium fluorescein that passed from the plasma into the brain was  
328 calculated as described previously<sup>43</sup>.

329

### 330 *Statistical analyses*

331 Sample sizes were calculated to detect differences of 15 % or more with a power of  
332 0.85 and α set at 5 %, calculations being informed by previously published data<sup>2,31</sup>. *In*  
333 *vitro* experimental data (except those for *in vitro* microarray experiments) are  
334 expressed as mean ± SEM, with a minimum of *n* = 3 independent experiments  
335 performed in triplicate for all studies. In all cases, normality of distribution was  
336 established using the Shapiro–Wilks test, followed by analysis with two-tailed  
337 Student's *t*-tests to compare two groups or, for multiple comparison analysis, 1- or 2-  
338 way ANOVA followed by Tukey's HSD *post hoc* test. Where data were not normally  
339 distributed, non-parametric analysis was performed using the Wilcoxon signed rank  
340 test. A *P* value of less than or equal to 5 % was considered significant. Differentially  
341 expressed genes were identified in microarray data using LIMMA (Ritchie *et al*, 2015);

342 *P* values were corrected for multiple testing using the Benjamini–Hochberg procedure  
343 (False Discovery Rate); a *P* value of less than or equal to 10 % was considered  
344 significant in this case; *n* = 5 for control, TMAO and TMA groups. Significantly  
345 differentially expressed genes ( $P_{\text{FDR}} < 0.1$ ) in RNAseq data (Supplementary Table 11)  
346 were identified using DESeq2 v1.22.1<sup>51</sup>.  
347

## 348 RESULTS

349 To provide an initial assessment of the effects of the methylamines TMA and TMAO  
350 upon the BBB we used a well-established *in vitro* BBB model, hCMEC/D3  
351 immortalised human cerebromicrovascular cell monolayers grown under polarising  
352 conditions on a Transwell filter, examining two key barrier properties: paracellular  
353 permeability to a protein-sized tracer and TEER. Exposure of hCMEC/D3 cells for 24 h  
354 to TMA (0-40  $\mu\text{M}$ ) caused a clear dose-dependent increase in paracellular  
355 permeability to 70 kDa FITC-dextran (Fig. 1A), with normal circulating levels (0.4  $\mu\text{M}$ )  
356 of TMA and upwards significantly enhancing permeability. In contrast, exposure for  
357 24 h to TMAO (0-4000  $\mu\text{M}$ ) caused a biphasic dose-dependent response (Fig. 1A),  
358 with normal circulating concentrations (40  $\mu\text{M}$ ) significantly reducing permeability to  
359 the tracer, an effect lost at 10-fold greater TMAO concentrations and reversed at 100-  
360 fold greater TMAO (4 mM), where a significant increase in paracellular permeability  
361 was apparent. In contrast, TMA had no effect upon TEER at any concentration studied,  
362 while TMAO enhanced TEER by approximately 65%, an effect that was notably dose-  
363 independent (Fig. 1B).

364  
365 The physical barrier that the BBB provides is only one aspect by which it separates  
366 the brain parenchymal environment from the periphery, equally important is the  
367 immunological barrier that it represents. To model this, we employed a simple system  
368 in which adhesion of CMFDA-labelled U937 monocytic cells to  $\text{TNF}\alpha$ -activated  
369 (10 ng/ml, 16 h) hCMEC/D3 monolayers was quantified in response to TMA or TMAO  
370 treatment. Treatment with a physiologically relevant concentration of TMA (0.4  $\mu\text{M}^{42}$ ,  
371 24 h post- $\text{TNF}\alpha$ ) had no effect on the density of adherent U937 cells, but exposure of  
372 hCMEC/D3 monolayers to physiological levels of TMAO (40  $\mu\text{M}^{42}$ , 24 h post- $\text{TNF}\alpha$ )  
373 significantly reduced U937 cell adhesion by approximately 50% compared to cultures  
374 stimulated with  $\text{TNF}\alpha$  alone (Fig. 1C).

375  
376 The endothelial cells of the BBB express numerous efflux transporter proteins that  
377 serve to limit entry of endogenous and exogenous molecules into the parenchyma,  
378 with BCRP and P-glycoprotein being two of the most important. Consequently, we  
379 examined whether treatment with TMA or TMAO affected function or expression of  
380 either of these two transporters. Using commercially available *in vitro* assays, neither

381 methylamine affected BCRP or P-glycoprotein activity across a wide concentration  
382 range (TMA: 4.9 nM to 10.8  $\mu$ M; TMAO 0.5  $\mu$ M to 1.08 mM) (Suppl. Fig. 2A-D).  
383 Similarly, treatment of hCMEC/D3 cells for 24 h with physiologically relevant  
384 concentrations of TMA (0.4  $\mu$ M) or TMAO (40  $\mu$ M) was without effect on cell surface  
385 expression of either BCRP or P-glycoprotein (Suppl. Fig. 2E-F).

386

### 387 *Methylamine-induced changes in gene expression*

388 Having identified significant TMA-/TMAO-induced functional changes in endothelial  
389 barrier characteristics *in vitro*, we undertook a microarray analysis of hCMEC/D3 cells  
390 treated with either TMA (0.4  $\mu$ M, 24 h) or TMAO (40  $\mu$ M, 24 h) to investigate the  
391 transcriptional changes underlying these effects. Treatment with TMA had a significant  
392 ( $P_{FDR}<0.1$ ) effect on 49 genes, with the expression of 39 upregulated and 10  
393 downregulated (Fig 2A, Supplementary Table 2). In contrast, treatment with TMAO  
394 had a significant ( $P_{FDR}<0.1$ ) effect on 440 genes with 341 upregulated and 99  
395 downregulated (Fig. 2B, Supplementary Table 3). *FMO3* gene expression was not  
396 affected by TMA or TMAO at the physiological concentrations employed (Suppl. Fig.  
397 3).

398

399 SPIA of the 440 TMAO-affected genes showed activation of the tight junction pathway  
400 ( $P = 0.031$ ), but significance was lost after correction for multiple testing  
401 (Supplementary Table 4). No pathways were shown to be activated or inactivated by  
402 the 49 TMA-affected genes (data not shown).

403

404 Gene ontology (GO) analysis was performed on TMA- and TMAO-regulated genes  
405 using Enrichr<sup>36,37</sup>. TMA up-regulated and down-regulated genes were significantly  
406 ( $P_{FDR}<0.2$ ) associated with processes indicative of a degree of cellular stress (Fig 2C,  
407 Supplementary Table 5, Supplementary Table 6). In contrast, genes up-regulated by  
408 TMAO treatment were associated with regulation of the cytoskeleton and cell  
409 morphology and with actin bundle formation ( $P_{FDR}<0.2$ ), whereas pathways associated  
410 with inflammatory signalling were down-regulated (Fig 2D, Supplementary Table 7,  
411 Supplementary Table 8).

412

413 We then assessed the topology of a directional network of the 440 TMAO-associated  
414 genes mapped onto all human KEGG pathways. In line with the GO analysis described

415 above, a number of genes of differing function were regulated by TMAO treatment,  
416 with two principal groupings being particularly evident, namely those associated with  
417 aspects of cellular metabolism and with regulation of actin cytoskeletal dynamics  
418 (Figure 2E). Finally, we compared the 19,309 genes represented on the microarray  
419 with a set of 203 genes<sup>2</sup> known to be associated with the BBB. While TMA treatment  
420 had no significant effects on expression of these genes (Supplementary Table 9),  
421 TMAO significantly ( $P_{\text{FDR}} < 0.1$ ) upregulated expression of four genes from this set  
422 associated with transporter proteins and barrier integrity (Table 1, Supplementary  
423 Table 10).

424

425

426 Given these transcriptional indications, and the fact that the restrictive properties of  
427 the BBB are largely governed by inter-endothelial cell tight junctions linked *via* the  
428 zonula occludens complex to the actin cytoskeleton<sup>52</sup>, we hypothesised that TMA and  
429 TMAO may affect barrier permeability through modification of the links between tight  
430 junctions and the actin cytoskeleton. Confocal immunofluorescence microscopy of  
431 hCMEC/D3 monolayers treated with a physiologically relevant concentration of TMA  
432 (0.4  $\mu\text{M}$ , 24 h) or TMAO (40  $\mu\text{M}$ , 24 h) revealed clear changes to both ZO-1 and  
433 fibrillar actin disposition within cells (Fig. 2F). Compared to untreated cells in which  
434 both ZO-1 and F-actin fibres clearly defined the cellular perimeter, cells treated with  
435 TMA exhibited a broken, patchy distribution of perimeter ZO-1 expression, and the  
436 appearance of marked cytoplasmic F-actin stress fibres. In contrast, cells treated with  
437 TMAO showed little change in ZO-1 distribution, but a marked enhancement of cortical  
438 F-actin fibre thickness and intensity.

439

440 *The actions of TMAO are mediated through annexin A1 signalling*

441 Of the four BBB-associated genes identified as upregulated by TMAO, *ANXA1* is of  
442 particular interest as we have previously shown this protein to regulate BBB tightness  
443 *in vitro* and *in vivo* through modulation of the actin cytoskeleton<sup>53</sup>. Examination of  
444 *ANXA1* expression in hCMEC/D3 cells revealed that while total cellular levels of the  
445 protein were not changed by either TMA (0.4  $\mu\text{M}$ , 24 h) or TMAO (40  $\mu\text{M}$ , 24 h)  
446 treatment (Fig. 3A), TMA significantly suppressed and TMAO significantly augmented  
447 medium *ANXA1* content (Fig. 3B), a finding of interest given that autocrine/paracrine  
448 effects are a major route of *ANXA1* action<sup>54</sup>.



449

450 To establish the importance of ANXA1 in mediating the effects of TMAO, we  
451 investigated the effects of its depletion through use of hCMEC/D3 clones stably  
452 transfected with shRNA sequences targeting *ANXA1* mRNA (Suppl. Fig. 1).  
453 Suppression of *ANXA1* expression significantly inhibited the effects of TMAO (40  $\mu$ M,  
454 24 h) upon both paracellular permeability and TEER (Fig. 3C-D), an effect not seen in  
455 cells bearing non-targeting scramble shRNA sequences. The actions of ANXA1 are  
456 mediated to a large extent through the G protein-coupled receptor formyl peptide  
457 receptor 2 (FPR2)<sup>55</sup>. Hence, we investigated how inclusion of a well-characterised  
458 antagonist to this receptor, WRW<sub>4</sub> (10  $\mu$ M, 10 min pre-treatment), would affect the  
459 functional response to TMAO. Pre-treatment with WRW<sub>4</sub> was able to significantly  
460 attenuate the effects of TMAO treatment on both TEER (Fig. 3E) and paracellular  
461 permeability (Fig. 3F), further indicating the role of ANXA1 signalling as the principal  
462 mediator of TMAO actions on hCMEC/D3 cells.

463

#### 464 *Acute beneficial effects of TMAO treatment in vivo*

465 While hCMEC/D3 endothelial cells are a widely used and generally representative  
466 model, they cannot reflect all aspects of the multicellular neurovascular unit that  
467 underlies BBB function, hence we investigated whether the beneficial effects of TMAO  
468 identified *in vitro* translate to an *in vivo* situation. Initial studies revealed that systemic  
469 administration of TMAO to wild-type male mice (1.8 mg/kg, i.p.) induced a time-  
470 dependent reduction in BBB permeability to the tracer Evans blue (2 % in saline, 100  
471  $\mu$ l, i.p.), with a significant reduction in dye extravasation to the brain parenchyma being  
472 apparent 2 h following TMAO administration, an effect lost at longer time-points (Fig.  
473 4A), presumably due to the relatively short plasma half-life of TMAO *in vivo*<sup>56</sup>. To  
474 further investigate this effect of TMAO, we employed a simple model of enhanced BBB  
475 permeability, namely acute peripheral administration of bacterial LPS<sup>31</sup>. Treatment  
476 with LPS (*E. coli* O111:B4, 3 mg/kg, i.p.) significantly enhanced intraparenchymal  
477 extravasation of Evans blue within 4 h, an effect significantly attenuated by subsequent  
478 treatment with TMAO (1.8 mg/kg, i.p.) 2 h post-LPS (Fig. 4B), further confirming a  
479 beneficial action of TMAO at physiological concentrations upon the BBB *in vivo*.

480

#### 481 *TMAO treatment rapidly alters brain transcriptional activity*

482 To investigate the wider actions of TMAO upon the brain we performed whole brain  
483 RNAseq transcriptomic analysis of wild-type male mice 2 h following TMAO  
484 administration (1.8 mg/kg i.p.). We identified 76 significantly differentially expressed  
485 genes ( $P_{FDR}<0.1$ ), with expression of 41 upregulated and 35 downregulated (Figure  
486 5A; Supplementary Table 11). KEGG pathway analysis using Enrichr identified a  
487 number of significantly regulated murine pathways (Figure 5B), including oxidative  
488 phosphorylation, Parkinson's disease and Alzheimer's disease. Closer analysis of  
489 regulated genes identified a number of general groupings (Figure 5C), with  
490 downregulated genes associated with the mitochondrial respiratory chain (*COX1*,  
491 *COX3*, *ATP6*, *ND4L*, *CYTB*, *ND1*, *ND3*, *ND4*, *ND6*) and ribosomal function (*mt-Rnr2*,  
492 *mt-Rnr1*, *Rps23rg1*) and upregulated genes associated with cellular or axonal growth  
493 (*Nme7*, *B3gat2*, *Fuz*, *Nefm*, *Basp1*, *Mtg1*, *Vps37a*, *Smim1*, *Araf*). Of the 203 BBB-  
494 associated human genes previously identified<sup>2</sup>, 197 had matches in our mouse brain  
495 data set. Here, two genes were identified as significantly differentially expressed at  
496  $P_{FDR}<0.1$ : reduced *Cpe* (carboxypeptidase E) and increased *App* (amyloid precursor  
497 protein) expression (Figure 5D; Supplementary Table 12).

498

#### 499 *Chronic low-dose TMAO treatment prevents LPS-induced BBB disruption and* 500 *memory impairment*

501 The fundamental role of the BBB is to protect the brain, preserving its homeostatic  
502 environment; damage to BBB integrity is therefore detrimental, and is believed to  
503 directly contribute towards cognitive impairment<sup>57-59</sup>. Having shown TMAO to exert a  
504 protective effect upon BBB function/integrity in response to acute inflammatory insult,  
505 we next examined whether a similar effect held true for chronic conditions, and  
506 whether this protection extended to cognition. TMAO was administered to male  
507 C57Bl/6J mice through drinking water (0.5 mg/ml) over 2 months, in combination with  
508 chronic low-dose LPS administration (0.5 mg/kg/week, i.p.) to model a mild  
509 inflammatory stress known to impact cognitive behaviour<sup>43</sup>. There were no differences  
510 in volumes of water drunk or, where relevant, final consumption of TMAO between any  
511 groups (Table 2). The serum inflammatory cytokines  $TNF\alpha$  and  $IL-1\beta$  were both  
512 nominally elevated in response to LPS treatment, although not reaching statistical  
513 significance, indicating a sub-clinical inflammatory response; TMAO had no effect on  
514  $TNF\alpha$  nor  $IL-1\beta$  levels (Suppl. Fig. 4). Notably, animals exposed to LPS exhibited a

515 significant reduction in body weight gain compared to their untreated counterparts, an  
516 effect reversed by TMAO treatment (Fig. 6A). Treatment with LPS increased  
517 cerebellar FITC extravasation, an effect that was prevented by TMAO treatment,  
518 although this did not reach statistical significance on *post hoc* analysis (Fig. 6B). To  
519 corroborate these findings, we investigated a second marker of impaired BBB integrity,  
520 confocal microscopic detection of brain perivascular IgG deposition. In comparison  
521 with sham-treated animals, exposure to LPS caused a significant accumulation of IgG  
522 in the perivascular compartment, an effect prevented by TMAO treatment (Fig. 6C).

523

524 The OFT confirmed neither LPS nor TMAO treatment affected motor function, with  
525 movement speed and distance travelled comparable across treatment groups (Fig.  
526 6D-E). Similarly, no effect was apparent on the proportion of time animals spent in the  
527 centre of the field, suggesting limited effects upon anxiety (Fig. 6F). Working memory,  
528 however, determined via NOR indicated a significant reduction in performance in  
529 animals exposed to LPS, a behavioural deficit notably prevented in animals co-treated  
530 with TMAO (Fig. 6G). In contrast, no effect of either LPS or TMAO treatment was  
531 apparent in the Y-maze spontaneous alternation task (Fig. 6H) or in distance travelled  
532 during this task (Fig. 6I), indicating no differences in spatial memory.

533

534

## 535 DISCUSSION

536

537 The relationship between the BBB and cognitive behaviour is complex and far from  
538 being fully understood, but it is clear from both human and animal studies that deficits  
539 in barrier integrity can exert a profound and deleterious effect upon memory, language  
540 and executive function<sup>60–63</sup>. Indeed, BBB impairment is among the first events to occur  
541 in the course of Alzheimer's disease, and may aggravate the pathological processes  
542 that underlie the condition<sup>64</sup>. Strategies to promote BBB function may thus have  
543 significant value in helping to protect the brain from progressive neurological diseases  
544 such as dementia. In this study we identify novel and distinct roles for the microbiome-  
545 associated dietary methylamines TMA and TMAO in regulating BBB function *in vitro*  
546 and *in vivo* and provide evidence that the beneficial action of TMAO upon the BBB  
547 coincides with similar protective effects upon cognition. These data reinforce the  
548 position of the cerebral vasculature as a major target for the gut-brain axis, and extend  
549 our knowledge of its interactions with microbial metabolites beyond SCFAs<sup>1,2</sup> to  
550 another major class of molecules, the dietary methylamines.

551

552 Notably, our data show that while both TMA and TMAO have activity upon the  
553 endothelium, there is a marked distinction between their effects despite their close  
554 structural similarity. TMA, the direct product of microbial choline, L-carnitine and  
555 TMAO metabolism in the upper gut<sup>5</sup>, had a deleterious effect upon the endothelium,  
556 disrupting cytoskeletal arrangement, inducing signs of metabolic stress and ultimately  
557 impairing endothelial barrier integrity. In contrast, TMAO, largely derived from hepatic  
558 FMO3-mediated oxidation of TMA taken up from the gut via the hepatic portal vein<sup>6</sup>,  
559 was beneficial for cerebral vascular integrity *in vitro* and *in vivo*. These differences  
560 suggest that host conversion of TMA (a gas) to TMAO (a stable metabolite) may be  
561 an effective detoxification pathway, emphasising the importance of host metabolic  
562 pathways in modulating communication in the gut-brain axis, and underlining the  
563 importance of using a systems-level approach to understand the interactions between  
564 the host and its resident microbiota.

565

### 566 *TMAO and cognitive function*

567 Numerous groups have investigated the putative relationship between TMAO and  
568 cognition following reports of an association between cerebrospinal fluid TMAO

569 content and Alzheimer's disease<sup>28</sup>, with negative correlations between plasma TMAO  
570 content and cognitive function having been identified in both clinical<sup>65-67</sup> and  
571 experimental<sup>29,68,69</sup> settings. Whether this relationship is truly deterministic remains  
572 unclear, however, as the role of the immediate precursor to TMAO, TMA, in cognition  
573 and vascular function has largely been overlooked. This omission may be important  
574 in light of studies reporting negative correlations between cognitive impairment and  
575 serum TMA<sup>70-72</sup> and our own data showing a potent detrimental effect of physiological  
576 levels of TMA upon the cerebrovascular endothelium *in vitro*. Given that TMA has  
577 been shown to be detrimental in contexts other than cognitive function<sup>22,23</sup>, the  
578 contribution that this metabolite plays in disease is evidently in need of closer attention.

579

580 Interpreting associations between circulating TMAO and cognition is further  
581 complicated by studies indicating that consumption of the TMAO precursors choline  
582 and L-carnitine can improve cognitive function<sup>24,25,73</sup>, evidence that patients with  
583 Parkinson's disease have lower circulating TMAO than healthy controls<sup>74</sup>, and more  
584 recent Mendelian randomisation analysis indicating that serum TMAO and Alzheimer's  
585 disease are not causally related<sup>75</sup>. Given this background, our data indicating that  
586 physiologically relevant concentrations of TMAO have positive effects upon both BBB  
587 integrity and cognition *in vivo* thus serve as a useful counterweight to population-level  
588 correlation studies. Interestingly, a number of previous interventional studies have  
589 been performed in mice, suggesting that substantially higher doses of TMAO may  
590 have detrimental effects upon learning and memory<sup>68,69,76</sup>, although as we and  
591 others<sup>77</sup> have identified dose-dependency in the effects of TMAO *in vitro*, it seems  
592 plausible that this may reflect a similar phenomenon *in vivo*. The importance of  
593 investigating the impact of TMAO under physiologically relevant conditions is further  
594 emphasised by a recent study showing TMAO treatment to impair novel object  
595 recognition in mice<sup>65</sup>, ostensibly an opposite finding to our data achieved with a similar  
596 dosing regimen. Importantly, however, mice in this study were maintained on a  
597 reduced choline diet, a condition known to alter hepatic metabolism<sup>78</sup>; what impact  
598 such changes might have on handling of (TMA and) TMAO by the body is unknown.  
599 These discrepancies may be instructive in guarding against incautious extrapolation  
600 of TMAO effects from healthy to diseased populations.

601

602 *Could TMAO contribute to the beneficial effects of a seafood-rich diet?*

603 Consumption of a diet rich in fish and other seafood, known to provide significant  
604 quantities of TMAO<sup>79</sup>, associates with a reduced risk of cognitive decline<sup>80,81</sup> and  
605 protection against cerebrovascular disease<sup>82</sup>. These effects have in large part been  
606 attributed to beneficial actions of the omega-3 polyunsaturated fatty acids<sup>83</sup>, although  
607 there is little evidence that their direct supplementation improves cognitive function<sup>84</sup>  
608 or stroke risk<sup>85</sup>. Here we provide evidence that another component of a seafood-rich  
609 diet, TMAO, has protective effects on the cerebral vasculature and upon cognition,  
610 indicating that broadening the scope of nutritional analyses beyond the omega-3 fatty  
611 acids may be worthwhile. In particular, we demonstrate that TMAO acts to stimulate  
612 endothelial secretion of a major regulator of BBB permeability, ANXA1<sup>53</sup>, leading *via*  
613 autocrine/paracrine activation of its receptor FPR2 to cytoskeletal actin rearrangement  
614 and enhanced BBB integrity.

615

616 This novel interaction between TMAO and ANXA1 may have implications beyond BBB  
617 integrity, given that ANXA1 is a major mediator of inflammatory resolution<sup>86</sup>, and  
618 highlighted by our finding that TMAO treatment inhibited model immune cell adhesion  
619 to activated endothelia *in vitro*. As with the link between a seafood-rich diet and  
620 cerebrovascular health, fish consumption has been associated with reduced  
621 inflammatory disease, again attributed primarily to a role for omega-3 fatty acids<sup>87</sup>.  
622 While it is too early to definitively claim an anti-inflammatory role for dietary  
623 methylamines, particularly given the opposing actions of TMA and TMAO, our data do  
624 suggest that investigation of the bioactive properties of seafood-rich diets should  
625 progress beyond omega-3 fatty acids to focus on other potential contributors.

626

## 627 *Conclusions*

628 Interest in the role played by the gut microbiota in communication through the gut-  
629 brain axis has grown dramatically in the last few years, with much attention focused  
630 on the mediating actions of microbe-derived metabolites<sup>88</sup>. While a number of studies  
631 have shown patterns in microbial metabolite production that associate with different  
632 brain functions<sup>89</sup>, detailed understanding of the role of individual molecules remains in  
633 its infancy, with defined roles characterised for only a subset of the many molecules  
634 known to be released by gut microbes. Here we show that the dietary methylamine  
635 TMAO can beneficially modulate both BBB integrity and cognitive function *in vivo*,  
636 providing direct mechanistic evidence for a positive role of this microbiome-associated

637 metabolite, and reinforce the position of the BBB as an interface in the gut-brain axis.  
638 Notably, the positive effects of TMAO that we report stand in contrast to previous work  
639 describing deleterious effects of TMAO exposure at high concentrations or under non-  
640 physiological conditions<sup>77</sup>, emphasising the importance of taking a holistic approach  
641 to understanding gut microbiota-host interactions.

642

643

644 **COMPETING INTERESTS**

645 The authors declare that they have no competing interests.

646

647 **ACKNOWLEDGEMENTS**

648 This work was funded by Alzheimer's Research UK Pilot Grant No. ARUK-PPG2016B-  
649 6. PREDEASY™ efflux transporter analysis kits were generously provided through the  
650 SOLVO Biotechnology Research and Academic Collaborative Transporter Studies  
651 (ReACTS) Program. This work used the computing resources of the UK MEDical  
652 BIOinformatics partnership – aggregation, integration, visualisation and analysis of  
653 large, complex data (UK MED-BIO), which was supported by the Medical Research  
654 Council (grant number MR/L01632X/1). SF was supported by Fundación Alfonso  
655 Martín Escudero. TS was supported by a bursary from the Imperial College London  
656 Undergraduate Research Opportunities Programme. LH, DV and SM designed the  
657 experiments. SM performed cellular assays and acute *in vivo* analyses. TS carried out  
658 the initial permeability and TEER assays. MAA performed IgG extravasation studies.  
659 ES produced and provided shRNA treated hCMEC/D3 clones. LH undertook all  
660 processing and analyses of transcriptomic data. RCG provided valuable insight and  
661 advice throughout the project. DV, MP, IR and MM performed the chronic *in vivo* LPS  
662 challenge study and undertook all analyses of behavioural data. SRC, ALC and SF  
663 contributed to preliminary animal work. LH, DV and SM wrote the manuscript. All  
664 authors read and approved the final version of the manuscript.

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- 897

898 **Table 1.** BBB-associated genes whose expression was upregulated upon exposure  
899 of hCMEC/D3 cells to TMAO.

900

<b>Gene</b>	<b>Entrez ID</b>	<b>Description</b>	<b>Log<sub>2</sub> fold change</b>	<b>Category</b>	<b>P<sub>FDR</sub></b>
<i>TFRC</i>	7037	Transferrin receptor	0.23	Transporter proteins	0.054
<i>ABCC4</i>	10257	ATP binding cassette subfamily C member 4	0.20	Transporter proteins	0.088
<i>ANXA1</i>	301	Annexin A1	0.16	Cell Adhesion/Junctional proteins/Cytoskeletal factors	0.088
<i>CDH2</i>	1000	Cadherin 2	0.31	Cell Adhesion/Junctional proteins/Cytoskeletal factors	0.095

901

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903



904 **Table 2.** Daily consumption of TMAO and water in mice chronically treated with TMAO  
905 and/or LPS.

906

907 Data are mean  $\pm$  standard deviation.

908

<b>Variable</b>	<b>Sham-treated mice</b>	<b>LPS-treated mice</b>	<b><i>P</i> value</b>
TMAO intake (mg/day/mouse)	2.79 $\pm$ 0.38	2.82 $\pm$ 0.25	0.7
TMAO intake (mg/kg/mouse)	85.0 $\pm$ 11.4	88.9 $\pm$ 7.9	0.14
Water consumption (ml/day/mouse)	5.57 $\pm$ 0.8	5.64 $\pm$ 0.5	0.48

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912

913 **FIGURE LEGENDS**

914 **Fig. 1.** Effects of TMAO and TMA on integrity of hCMEC/D3 cell monolayers. (A)  
915 Assessment of paracellular permeability of hCMEC/D3 monolayers to a 70 kDa FITC-  
916 dextran tracer following treatment for 24 h with varying doses of TMA (0.4 – 40  $\mu$ M) or  
917 TMAO (40 – 4000  $\mu$ M). Data are expressed as mean  $\pm$  s.e.m.,  $n=4$  independent  
918 experiments. (B) Assessment of TEER of hCMEC/D3 monolayers to a 70kDa FITC-  
919 dextran tracer following treatment for 24 h with varying doses of TMA (0.4 – 40  $\mu$ M) or  
920 TMAO (40 – 4000  $\mu$ M). Data are expressed as mean  $\pm$  s.e.m.,  $n=4$  independent  
921 experiments. (C) Adhesion of U937 monocytic cells to TNF $\alpha$ -stimulated hCMEC/D3  
922 monolayers (10 ng/ml, 16 h) that had been treated or not for 24 h with 0.4  $\mu$ M TMA or  
923 40  $\mu$ M TMAO. Data are expressed as mean  $\pm$  s.e.m.,  $n=3$  independent experiments.

924  
925 **Fig. 2.** Effects of TMA and TMAO on gene expression in hCMEC/D3 cells. (A)  
926 Heatmap showing expression of the 49 genes found to be significantly ( $P_{FDR}<0.1$ )  
927 differentially expressed upon exposure of hCMEC/D3 cells to 0.4  $\mu$ M TMA ( $n=5$  per  
928 group). (B) Heatmap showing expression of the 440 genes found to be significantly  
929 ( $P_{FDR}<0.1$ ) differentially expressed upon exposure of hCMEC/D3 cells to 40  $\mu$ M TMAO  
930 ( $n=5$  per group). (C) Biological processes associated with genes found to be  
931 significantly upregulated ( $n=39$ ) or downregulated ( $n=10$ ) upon exposure of cells to  
932 TMA. (D) Biological processes of genes found to be significantly upregulated ( $n=341$ )  
933 or downregulated ( $n=99$ ) upon exposure of cells to TMAO. Images in (C, D) shown  
934 based on Enrichr  $P$  value ranking from GO analysis. (E) Topological analysis of the  
935 KEGG networks associated with the 440 genes whose expression was significantly  
936 affected upon exposure of cells to TMAO (blue, significantly downregulated; red,  
937 significantly upregulated); genes of similar cellular role are highlighted. (F) Confocal  
938 microscopic analysis of expression of fibrillar actin (F-actin) and the tight junction  
939 component zonula occludens-1 (ZO-1) in hCMEC/D3 cells following treatment for 24  
940 h with 0.4  $\mu$ M TMA or 40  $\mu$ M TMAO. Images are representative of at least three  
941 independent experiments.

942  
943 **Fig. 3.** Annexin A1 (ANXA1) signalling mediates effects of TMAO on hCMEC/D3 cells.  
944 (A) Total cellular expression of ANXA1 in hCMEC/D3 cells treated for 24 h with 0.4  
945  $\mu$ M TMA or 40  $\mu$ M TMAO. Data are expressed as mean  $\pm$  s.e.m.,  $n=5-7$  independent

946 experiments. (B) Medium ANXA1 content of hCMEC/D3 monolayers treated for 24 h  
947 with 0.4  $\mu$ M TMA or 40  $\mu$ M TMAO. Data are expressed as mean  $\pm$  s.e.m.,  $n=3$   
948 independent experiments. (C) Assessment of paracellular permeability of monolayers  
949 of wild-type hCMEC/D3 cells, or hCMEC/D3 cells stably transfected with either a  
950 scramble shRNA sequence or an shRNA sequence targeting ANXA1 to a 70kDa FITC-  
951 dextran tracer following treatment for 24 h with 40  $\mu$ M TMAO. Data are expressed as  
952 mean  $\pm$  s.e.m.,  $n=4$  independent experiments. (D) Assessment of TEER of  
953 monolayers of wild-type hCMEC/D3 cells, or hCMEC/D3 cells stably transfected with  
954 either a scramble shRNA sequence or an shRNA sequence targeting ANXA1 following  
955 treatment for 24 h with 40  $\mu$ M TMAO. Data are expressed as mean  $\pm$  s.e.m.,  $n=4$   
956 independent experiments. (E) Assessment of paracellular permeability of hCMEC/D3  
957 cells to a 70kDa FITC-dextran tracer following treatment for 24 h with 40  $\mu$ M TMAO,  
958 with or without 10 min pre-treatment with the FPR2 antagonist WRW<sub>4</sub> (10  $\mu$ M). Data  
959 are expressed as mean  $\pm$  s.e.m.,  $n=3$  independent experiments. (F) Assessment of  
960 TEER of hCMEC/D3 cells following treatment for 24 h with 40  $\mu$ M TMAO, with or  
961 without 10 min pre-treatment with the FPR2 antagonist WRW<sub>4</sub> (10  $\mu$ M). Data are  
962 expressed as mean  $\pm$  s.e.m.,  $n=3$  independent experiments.

963

964 **Fig. 4.** Acute treatment with TMAO promotes BBB integrity *in vivo*. (A) Extravasation  
965 of Evans blue dye into brain parenchyma over a 1 h period in 2-month-old male  
966 C57Bl/6J mice following i.p. injection of 1.8 mg/kg TMAO for 2 h, 6 h or 24 h vs. a  
967 saline injected control. Data are normalised to plasma Evans blue content, and are  
968 expressed as mean  $\pm$  s.e.m.,  $n=5-6$  mice. (B) Extravasation of Evans blue dye into  
969 brain parenchyma over a 1 h period in 2-month-old male C57Bl/6J mice following i.p.  
970 injection of saline or E. coli O111:B4 LPS (3 mg/kg) with or without subsequent i.p.  
971 injection of 1.8 mg/kg TMAO according to the schedule shown. Data are normalised  
972 to plasma Evans blue content, and are expressed as mean  $\pm$  s.e.m.,  $n=4-6$  mice.

973

974 **Fig. 5.** Acute exposure of mice to TMAO significantly alters the whole brain  
975 transcriptome. (A) Heatmap showing expression of the 76 genes found to be  
976 significantly ( $P_{FDR}<0.1$ ) differentially expressed in the mouse brain after 2 h exposure  
977 to 1.8 mg/kg TMAO ( $n=3$  per group). Data were scaled by row. (B) Over-representation  
978 analysis (Enrichr) showing KEGG pathways associated with the 76 genes. (C)

979 Comparative analysis of significantly differentially expressed genes identified  
980 groupings associated with distinct biological functions. (D) Among the 197 BBB-  
981 specific genes identified in the data set, only *App* and *Cpe* were significantly  
982 ( $P_{FDR}<0.1$ ) differentially expressed in the mouse brain after 2 h exposure to TMAO.  
983 Data are shown as mean  $\pm$  s.d,  $n=3$  per group. Individual data points are not shown  
984 due to the negligible values of the s.d.

985

986 **Fig. 6.** Effect of long-term TMAO exposure on BBB integrity and cognitive function of  
987 mice. (A) Body weight gain in mice treated with TMAO through their drinking water  
988 (0.5 mg/ml) over 2 months, combined with a chronic low dose administration of LPS  
989 (0.5 mg/kg/week, i.p.). Data are expressed as mean  $\pm$  s.e.m.,  $n=8$  mice, columns with  
990 different letters are significantly different at  $P<0.05$ . (B) Cerebellar permeability index  
991 to sodium fluorescein 2h following administration in animals previously treated with  
992 TMAO through their drinking water (0.5 mg/ml) over 2 months, combined with a  
993 chronic low dose administration of LPS (0.5 mg/kg/week, i.p.). Data are expressed as  
994 mean  $\pm$  s.e.m.,  $n=8$  mice, columns with different letters are significantly different at  
995  $P<0.05$ . (C) Typical confocal microscopic images of perivascular IgG deposition in  
996 male C57Bl/6J mice treated with TMAO through their drinking water (0.5 mg/ml) over  
997 2 months, combined with a chronic low dose administration of LPS (0.5 mg/kg/week,  
998 i.p.). *Griffonia simplicifolia* isolectin B<sub>4</sub> (red) defines endothelial cells, areas of IgG  
999 deposition (white) are highlighted by arrow heads. (D) Distance travelled, (E)  
1000 movement speed and (F) percentage of time in the centre as measured in the OFT in  
1001 animals previously treated with TMAO through their drinking water (0.5 mg/ml) over 2  
1002 months, combined with a chronic low dose administration of LPS (0.5 mg/kg/week,  
1003 i.p.). Data are expressed as mean  $\pm$  s.e.m.,  $n=8$  mice. (G) Novel object discrimination  
1004 index, calculated as described in Methods, of animals previously treated with TMAO  
1005 through their drinking water (0.5 mg/ml) over 2 months, combined with a chronic low  
1006 dose administration of LPS (0.5 mg/kg/week, i.p.). Data are expressed as mean  $\pm$   
1007 s.e.m.,  $n=8$  mice, columns with different letters are significantly different at  $P<0.05$ .  
1008 (H) Percentage of spontaneous alternation and (I) total distance travelled in the Y-  
1009 maze test for animals previously treated with TMAO through their drinking water (0.5  
1010 mg/ml) over 2 months, combined with a chronic low dose administration of LPS (0.5  
1011 mg/kg/week, i.p.). Data are expressed as mean  $\pm$  s.e.m.,  $n=8$  mice.

1012

1013 **Supplementary Fig. 1.** Confirmation of ANXA1 targeting and knock-down in  
1014 hCMEC/D3 cells stably transfected with appropriate shRNA sequences. (A) Typical  
1015 flow cytometric profiles of wild-type cells, cells transfected with a scramble shRNA  
1016 sequence and cells transfected with an ANXA1-targeting shRNA sequence, alongside  
1017 unstained and second antibody controls. (B) Expression of ANXA1 in wild-type  
1018 hCMEC/D3 cells, cells transfected with a scramble shRNA sequence and cells  
1019 transfected with an ANXA1-targeting shRNA sequence. Data are expressed as mean  
1020  $\pm$  s.e.m.,  $n=4$  independent experiments.

1021  
1022 **Supplementary Fig. 2.** Neither TMAO nor TMA treatment affected the major  
1023 endothelial efflux transporters BCRP or P-glycoprotein. (A-D) *In vitro* analysis revealed  
1024 no significant effects of either TMAO or TMA upon BCRP (A, C) or P-glycoprotein (B,  
1025 D) activity. Dashed lines represent Loess regression fits, with shading representing  
1026 95% confidence intervals. (E) hCMEC/D3 cell surface expression of BCRP was  
1027 unaffected by 24 h exposure to TMA (0.4  $\mu$ M) or TMAO (40  $\mu$ M). Data are expressed  
1028 as mean  $\pm$  s.e.m.,  $n=3$  independent experiments. (F) hCMEC/D3 cell surface  
1029 expression of P-glycoprotein was unaffected by 24 h exposure to TMA (0.4  $\mu$ M) or  
1030 TMAO (40  $\mu$ M). Data are expressed as mean  $\pm$  s.e.m.,  $n=3$  independent experiments.

1031  
1032 **Supplementary Fig. 3.** *In vitro* (hCMEC/D3 cell) *FMO3* gene expression is unaffected  
1033 by exposure to TMA (0.4  $\mu$ M) or TMAO (40  $\mu$ M). Dark blue lines represent the standard  
1034 deviation (+/-). There was no statistically significant difference between TMA and the  
1035 control, nor between TMAO and the control (Supplementary Tables 2 and 3).  
1036 Individual data points are not shown due to the negligible values of the standard  
1037 deviations.

1038  
1039 **Supplementary Fig. 4.** Neither chronic low-dose LPS nor TMAO treatment  
1040 significantly increases serum inflammatory cytokines. (A) Serum TNF $\alpha$  concentrations  
1041 in male C57Bl/6J mice treated with TMAO through their drinking water (0.5 mg/ml)  
1042 over 2 months, combined with a chronic low dose administration of LPS (0.5  
1043 mg/kg/week, i.p.). Data are expressed as mean  $\pm$  s.e.m.,  $n=7-8$  mice. (B) Serum IL-  
1044 1 $\beta$  concentrations in male C57Bl/6J mice treated with TMAO through their drinking  
1045 water (0.5 mg/ml) over 2 months, combined with a chronic low dose administration of

1046 LPS (0.5 mg/kg/week, i.p.). Data are expressed as mean  $\pm$  s.e.m.,  $n=7-8$  mice.

1047

1048 **Supplementary Table 1.** Normalized microarray data for hCMEC/D3 treated or not  
1049 with TMA (0.4  $\mu$ M) or TMAO (40  $\mu$ M)

1050

1051 **Supplementary Table 2.** Differential gene expression for control compared with TMA  
1052 treatment, as assessed using LIMMA

1053

1054 **Supplementary Table 3.** Differential gene expression for control compared with  
1055 TMAO treatment, as assessed using LIMMA

1056

1057 **Supplementary Table 4.** KEGG-based SPIA of genes whose expression was  
1058 significantly affected by TMAO

1059

1060 **Supplementary Table 5.** GO (biological process) analysis of TMA-up-regulated  
1061 genes as assessed using Enrichr

1062

1063 **Supplementary Table 6.** GO (biological process) analysis of TMA-down-regulated  
1064 genes as assessed using Enrichr

1065

1066 **Supplementary Table 7.** GO (biological process) analysis of TMAO-up-regulated  
1067 genes as assessed using Enrichr

1068

1069 **Supplementary Table 8.** GO (biological process) analysis of TMAO-down-regulated  
1070 genes as assessed using Enrichr

1071

1072 **Supplementary Table 9.** Differential gene expression of BBB-associated genes (*in*  
1073 *vitro* TMA treatment)

1074

1075 **Supplementary Table 10.** Differential gene expression of BBB-associated genes (*in*  
1076 *vitro* TMAO treatment)

1077

1078 **Supplementary Table 11.** Differential gene expression in mouse brain for control  
1079 compared with TMAO treatment, as assessed using DESeq2

1080

1081 **Supplementary Table 12.** Differential expression of BBB-associated genes in mouse  
1082 brain for control compared with TMAO treatment, as assessed using DESeq2

1083

Figure 1

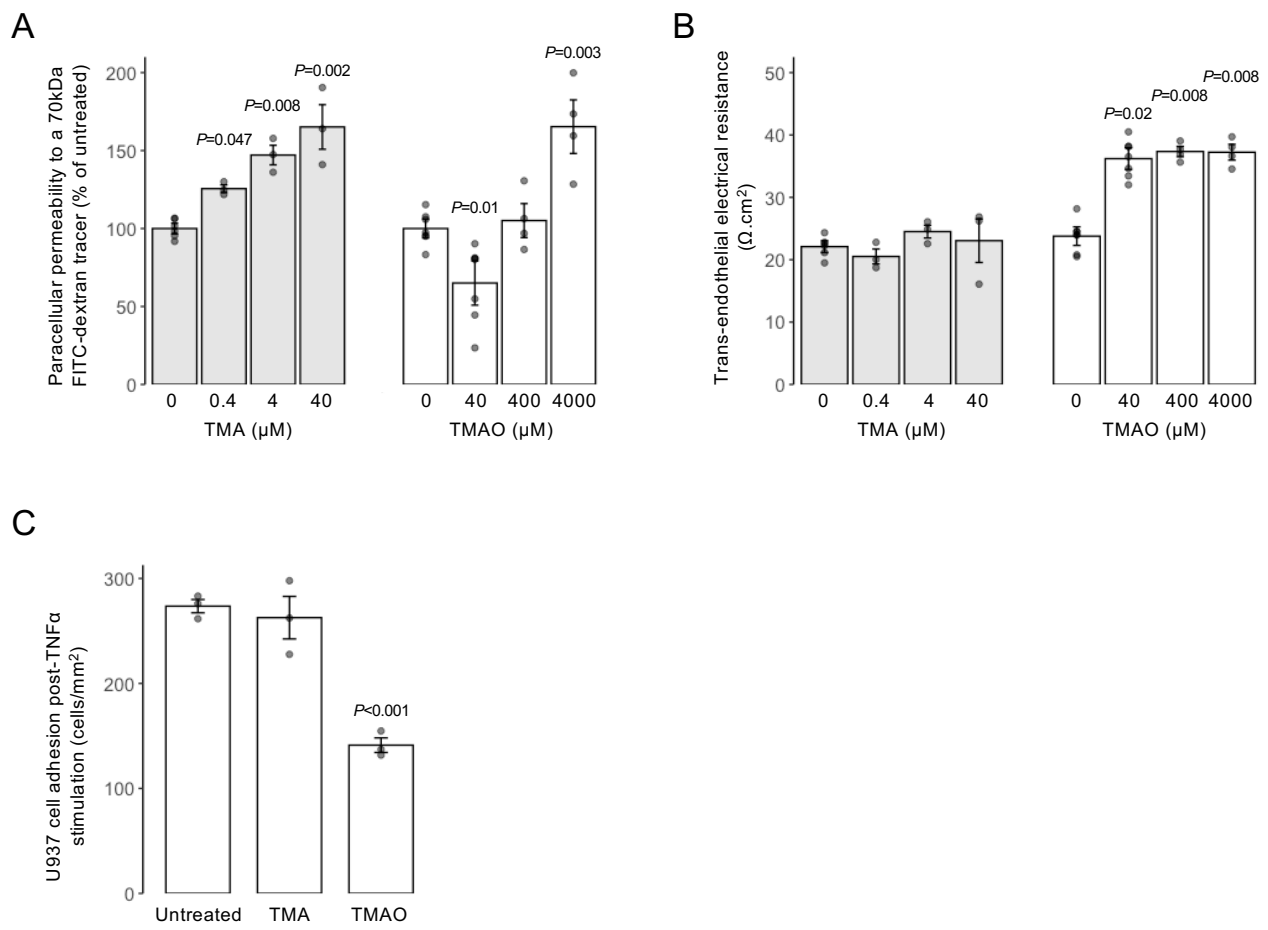




Figure 2

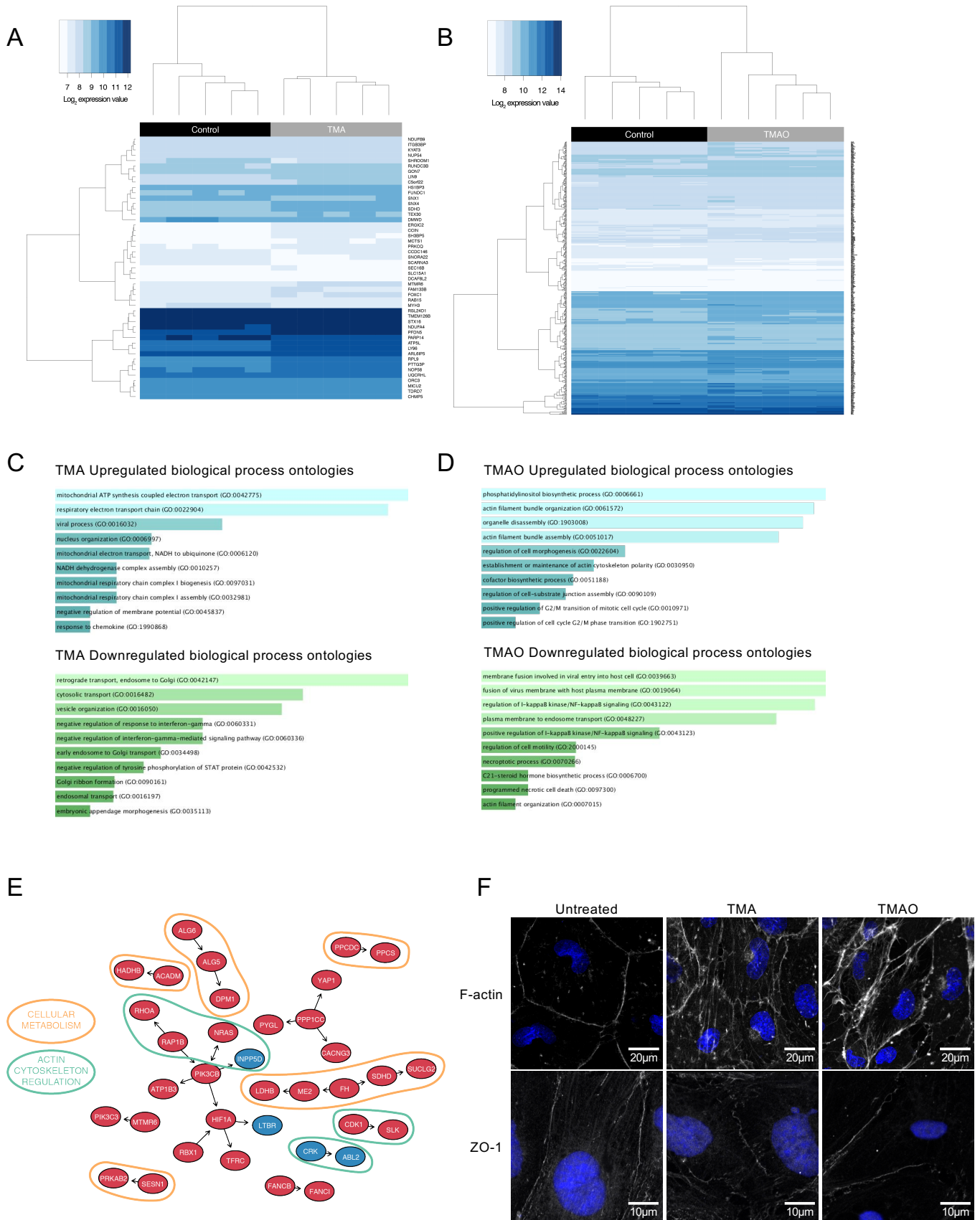


Figure 3

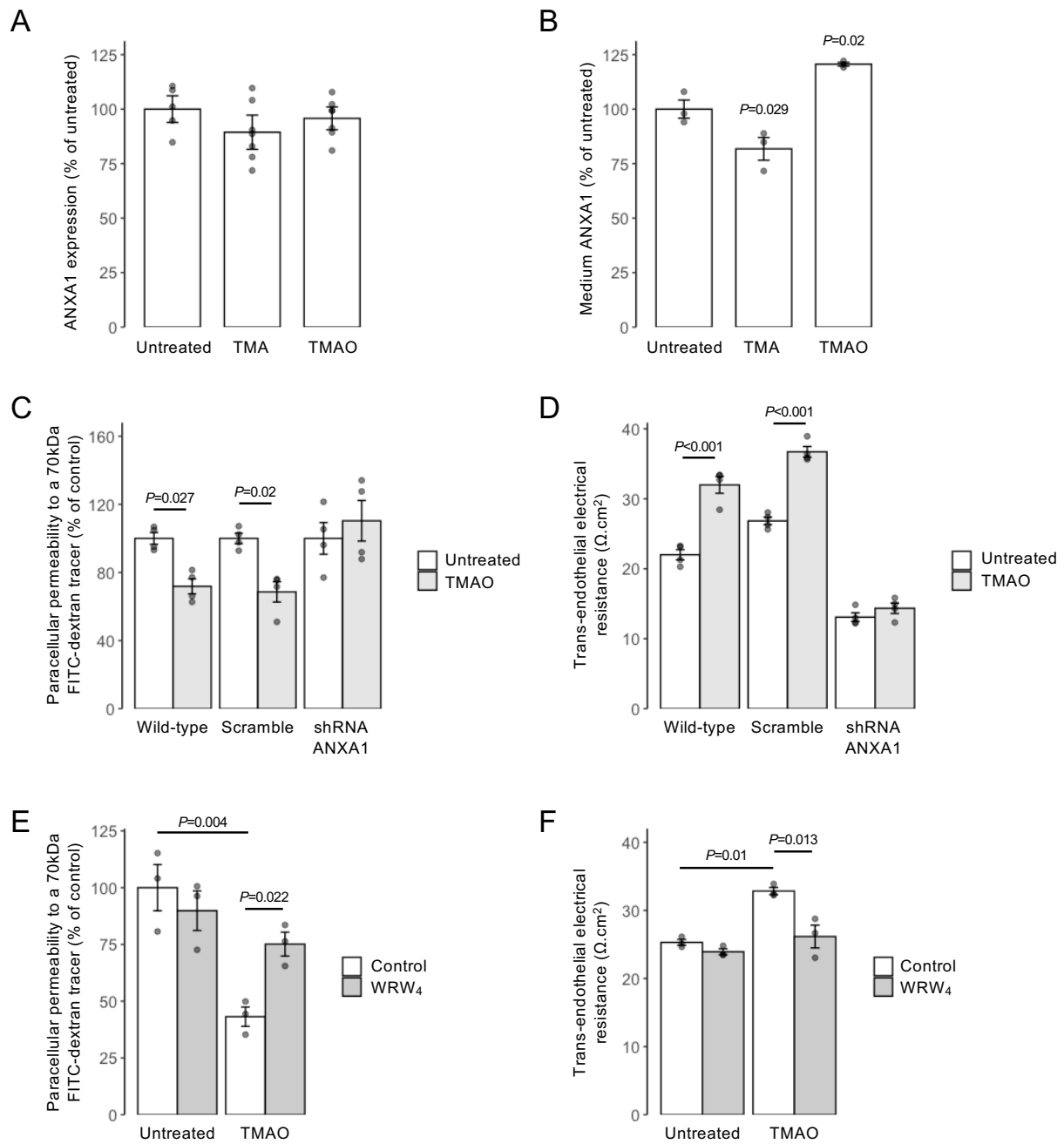


Figure 4

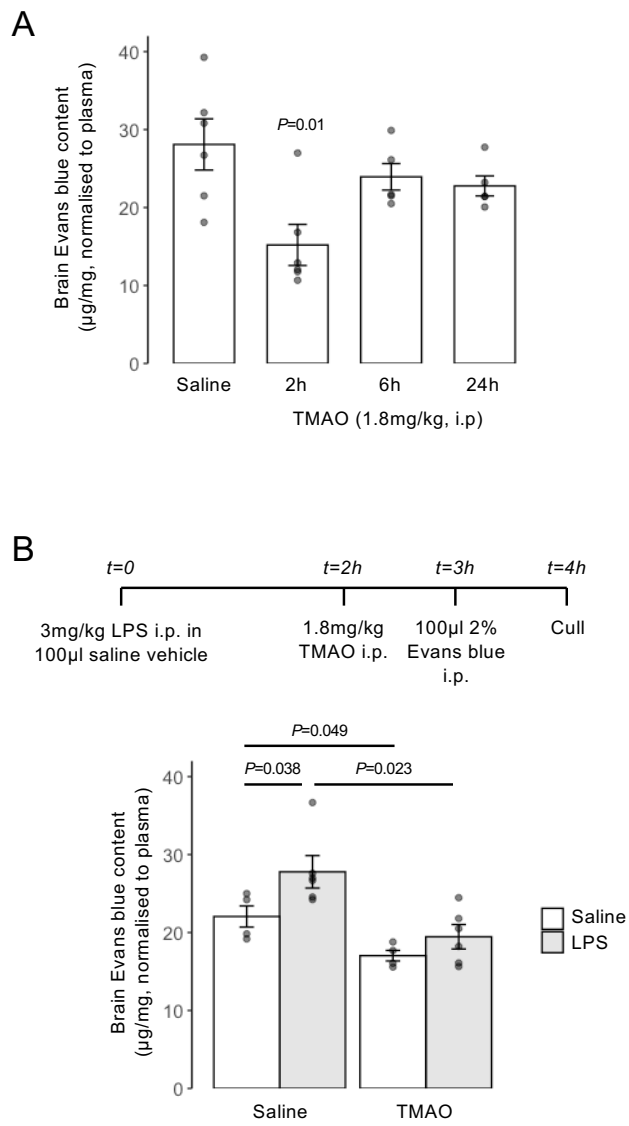


Figure 5

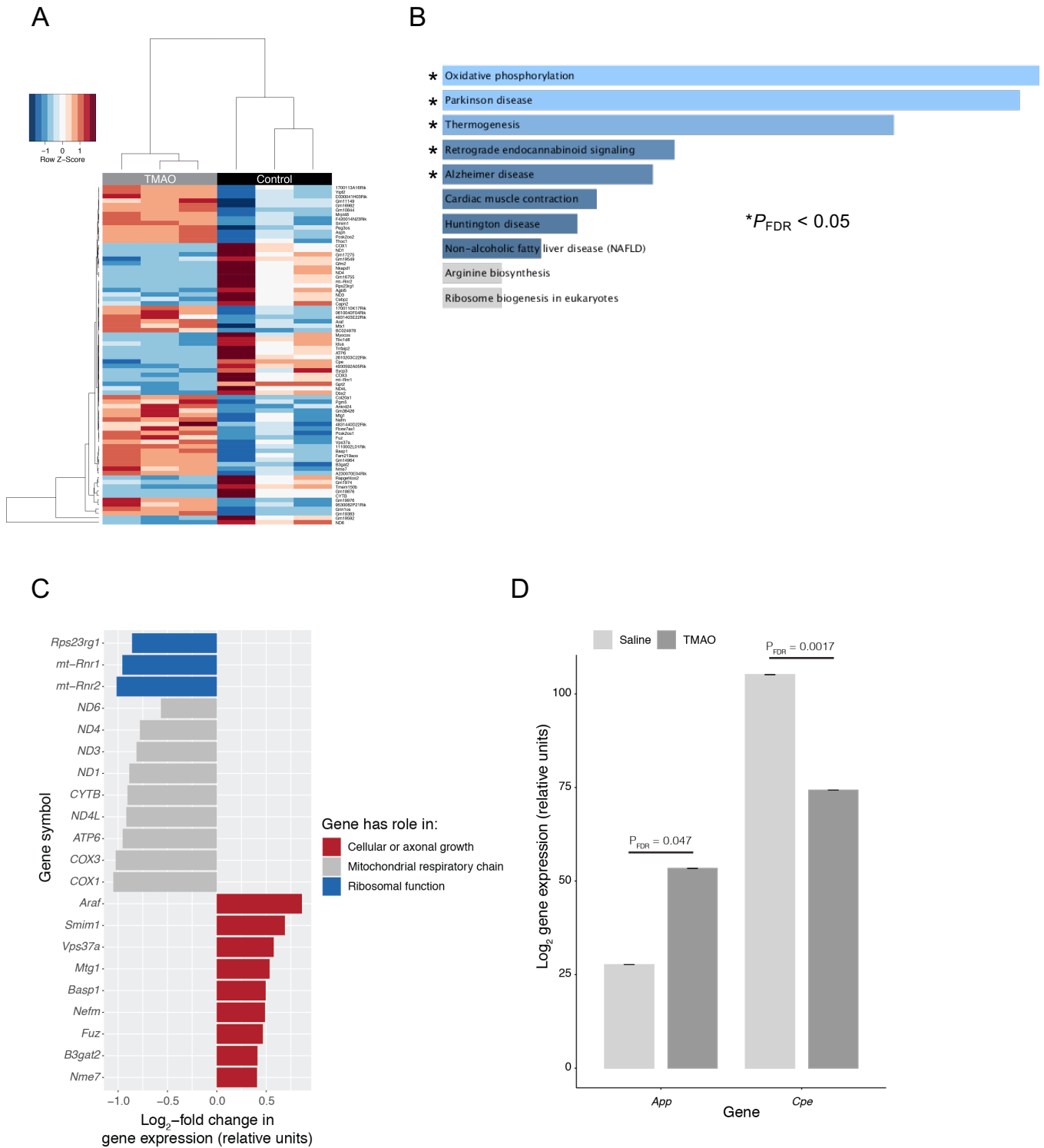
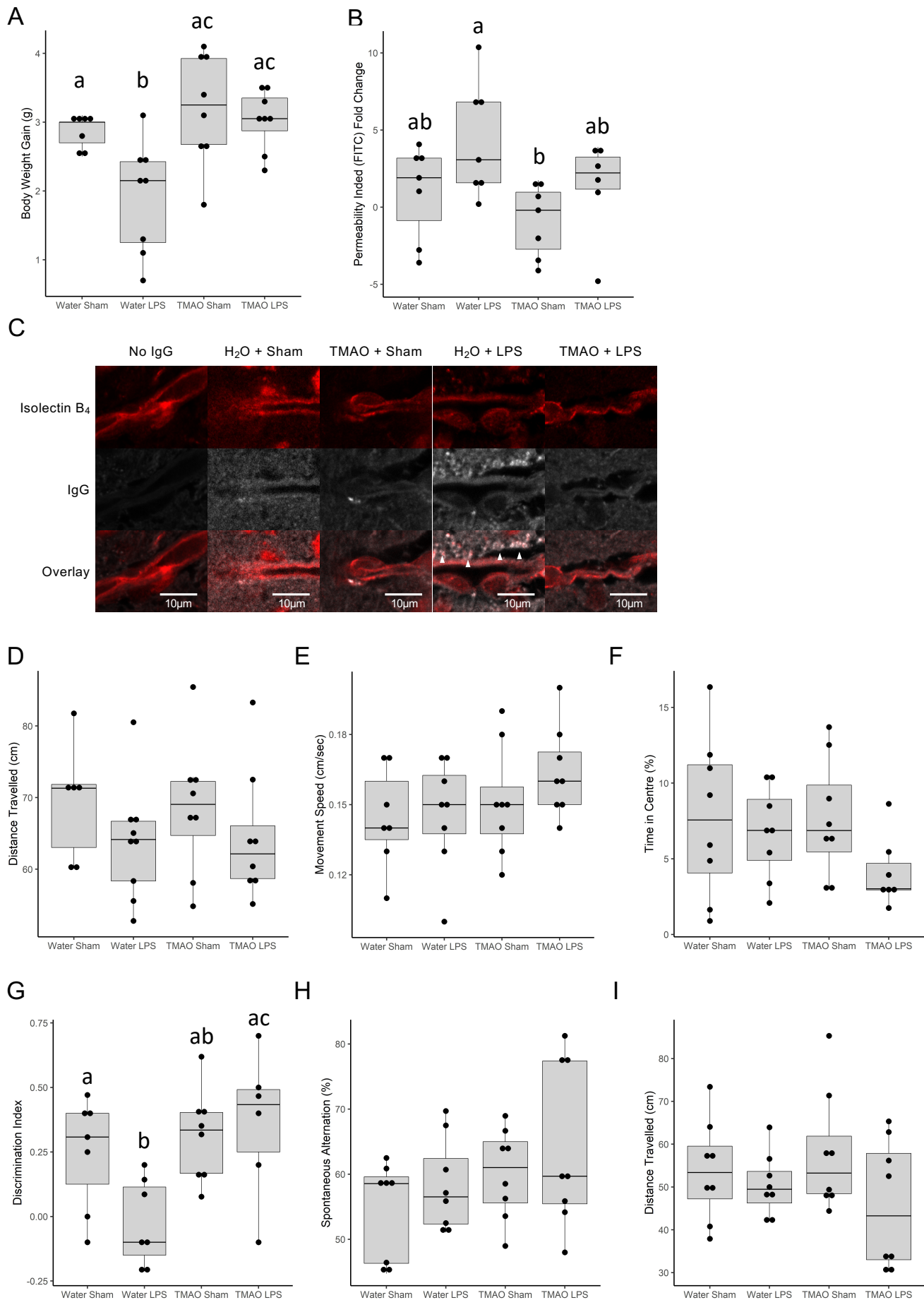
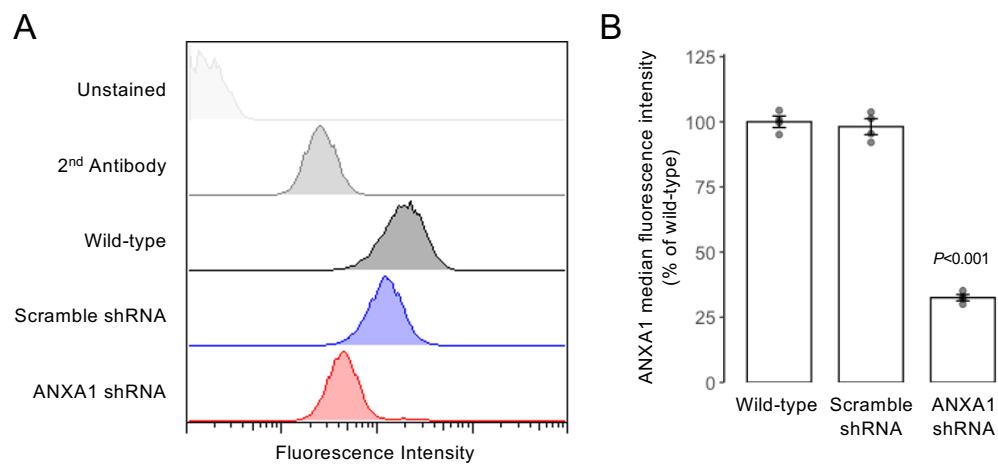


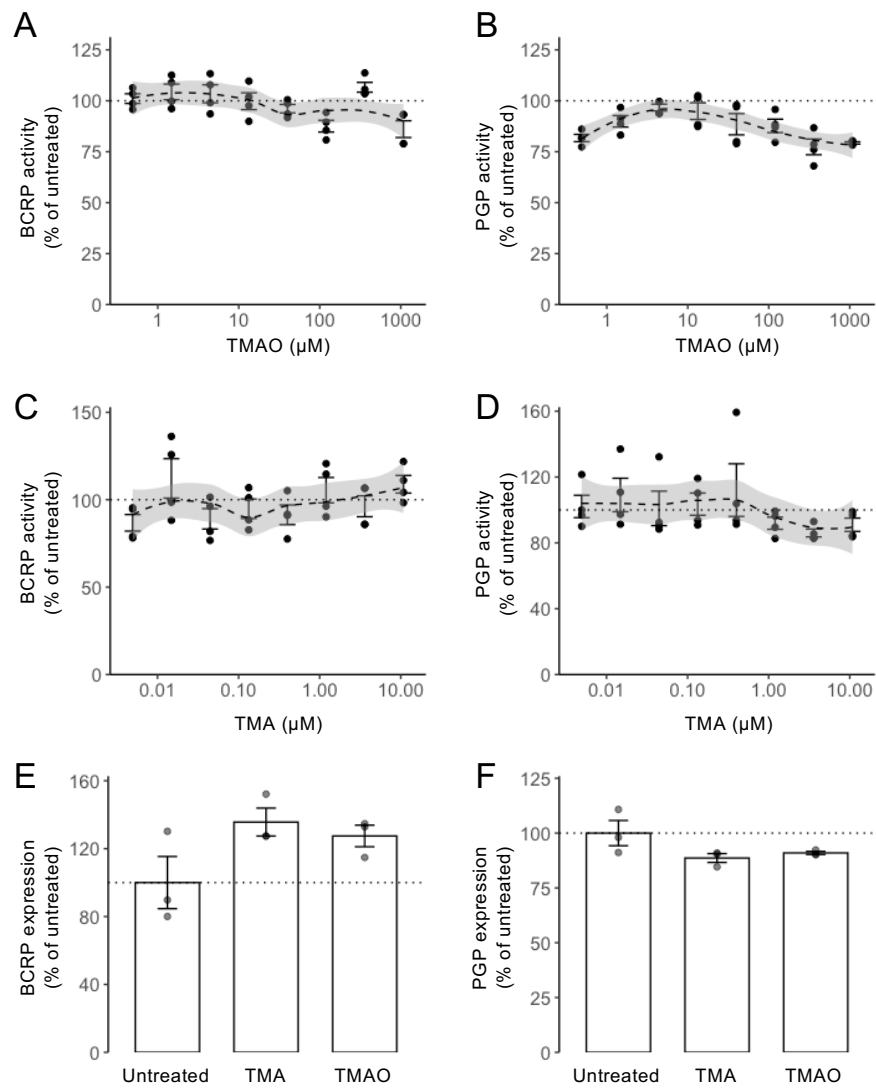
Figure 6



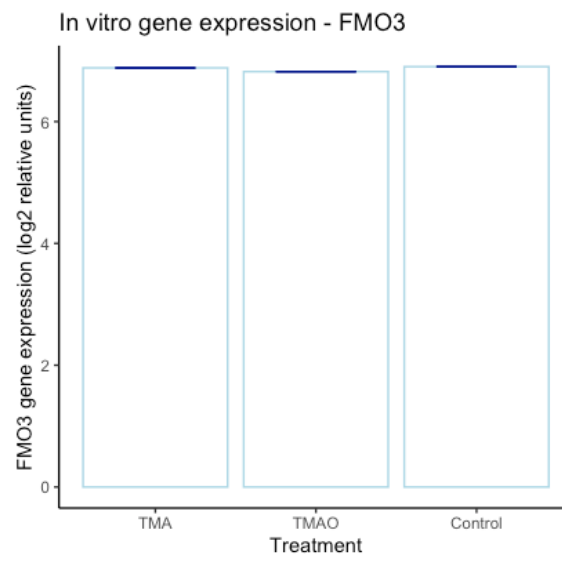
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3





Supplementary Figure 4

