

1 Age-linked suppression of lipoxin A4 mediates cognitive deficits in
2 mice and humans

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31

32 **Abstract**

33 Age increases the risk for cognitive impairment and is the single major risk factor for
34 Alzheimer's disease (AD), the most prevalent form of dementia in the elderly. The
35 pathophysiological processes triggered by aging that render the brain vulnerable to
36 dementia involve, at least in part, changes in inflammatory mediators. Here we show
37 that lipoxin A4 (LXA4), a lipid mediator of inflammation resolution known to stimulate
38 endocannabinoid signaling in the brain, is reduced in the aging central nervous system.
39 We demonstrate that genetic suppression of 5-lipoxygenase (5-LOX), the enzyme
40 mediating LXA4 synthesis, promotes learning impairment in mice. Conversely,
41 administration of exogenous LXA4 attenuated cytokine production and memory loss
42 induced by inflammation in mice. We further show that cerebrospinal fluid LXA4 is
43 reduced in patients with dementia and positively associates with memory performance,
44 brain-derived neurotrophic factor (BDNF), and AD-linked amyloid- β . Our findings
45 suggest that reduced LXA4 levels may lead to vulnerability to age-related cognitive
46 disorders and that promoting LXA4 signaling may comprise an effective strategy to
47 prevent early cognitive decline in AD.

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55 **Introduction**

56 Inflammation constitutes an essential defensive mechanism that is dysregulated
57 in aging and in several chronic and neurodegenerative disorders, including Alzheimer's
58 disease (AD) ^{1, 2}. Accordingly, a chronic imbalance favoring disproportionate pro-
59 inflammatory responses in the brain and periphery are thought to mediate age-related
60 cognitive decline and AD pathogenesis ³⁻⁹.

61 Proper control of inflammatory responses depends on resolution processes,
62 which are controlled by lipid mediators known as resolvins, such as lipoxins ¹⁰. Lipoxin
63 A4 (LXA4) is an eicosanoid derived from arachidonic acid through transcellular
64 metabolic pathways that depend on the enzymatic activity of 5-lipoxygenase (5-LOX) ¹⁰⁻
65 ¹². In the periphery, LXA4 activates the G-protein-coupled receptor ALX to modulate
66 gene expression towards resolution of inflammation and to promote immune cell
67 recruitment to the site of infection or damage ¹⁰. Putative effects of LXA4 in the brain
68 have long remained elusive, due to the low expression of ALX receptors in the central
69 nervous system (CNS) ¹³.

70 We and others have suggested that LXA4 protects the central nervous system
71 against injuries ¹⁴⁻¹⁸, including amyloid- β (A β) toxicity in mice ^{15, 19, 20}. However, while
72 the processes that propagate cytokine-mediated inflammation in AD have been
73 thoroughly investigated, very little is known about the impact and mechanisms of LXA4
74 on brain function. Given that ALX receptors are poorly expressed in the central nervous
75 system ¹³, other mechanisms should be responsible for the brain actions of LXA4. We
76 have previously demonstrated that LXA4 binds to CB1 receptors in the brain to
77 stimulate endocannabinoid signaling ¹⁹. These findings raise the prospect that LXA4

78 might be relevant to complex brain functions and that LXA4 signaling might be impaired
79 in age-related cognitive diseases linked to aberrant inflammation, such as AD.

80 Herein we report that mammalian neurons and microglia produce considerable
81 levels of LXA4. We further demonstrate that brain and peripheral LXA4, as well as
82 memory, decline with aging and that genetic suppression of 5-LOX results in memory
83 impairment in mice. Conversely, administration of exogenous LXA4 attenuated cytokine
84 production and memory loss induced by an inflammatory stimulus in mice. We finally
85 present results from a human study demonstrating that cerebrospinal fluid (CSF) LXA4
86 levels are reduced in dementia. Furthermore, LXA4 correlates with memory
87 performance, A β and brain-derived neurotrophic factor (BDNF) in the human CSF.
88 Altogether, these results support a protective role for LXA4 in the brain that is
89 dysregulated in aging and, to a greater extent, in dementia.

90

91 **Methods**

92

93 **Ethics**

94 All mouse experiments were performed under protocols approved and supervised by
95 the Institutional Animal Care and Use Committee of Federal University of Rio de Janeiro
96 (CEUA-UFRJ) (protocol no. IBQM058). Experimental procedures involving human
97 clinical data and CSF samples were approved by the Institutional Review Board (IRB) of
98 Copa D'Or Hospital (protocol #47163715.0.0000.5249). Written informed consent was
99 obtained from all volunteers. Samples were anonymized and measurements were

100 performed by trained investigators in a blinded fashion. All studies have been performed
101 according to the national and international ethical regulations and standards.

102

103 **iPSC cultures**

104 Three different lines of human induced pluripotent stem cells were employed in this
105 study - GM23279A iPSC line, obtained commercially from Coriell Institute Biobank; CF2
106 line, generated from fibroblasts; and C15 line, obtained from urine cells. Cells were
107 reprogrammed using the CytoTune 2.0 Sendai Reprogramming kit (Thermo Fisher
108 Scientific, USA) and characterization of the reprogrammed cell lines was conducted by
109 immunostaining of both iPSCs colonies and iPSCs- derived embryoid bodies for self-
110 renewal and three germ layer markers as described^{21, 22}. iPSCs were used to generate
111 human neural progenitor cells (NPCs) by induction with retinoic acid (RA) for 18 days²³,
112²⁴. When neural tube-like structures emerged, they were collected and re-plated on
113 adherent dishes treated with 10 µg/mL Poly-L-ornithine and 2.5 µg/mL laminin (Thermo
114 Fisher Scientific). After passages using Accutase, homogenous cultures were obtained
115²⁵. NPCs were maintained and grown in DMEM/F-12 supplemented with N2, B-27, 25
116 ng/mL bFGF and 20 ng/mL EGF (Thermo Fisher Scientific), with medium changes
117 every other day. The astrocyte cultures and mixed neuronal cultures were obtained from
118 the differentiation of neural stem cells (NSCs), as described²⁶.

119

120 **Primary cell cultures**

121 Primary astrocyte, microglial and neuronal cultures were obtained from Swiss mice.
122 Brain tissue was dissociated into single cells in DMEM-F12 (Invitrogen; Carlsbad, CA,

123 USA) supplemented with glutamine (2 mM), penicillin and streptomycin (0.5 mg/mL,
124 HyClone), amphotericin B (0.65 μ M, Sigma Aldrich, St. Louis, MO, USA) and 10% fetal
125 bovine serum (FBS, Invitrogen; Invitrogen, Carlsbad, CA, USA). Cells were plated in 25
126 cm² bottles pre-treated with poly-L-lysine (Sigma Aldrich, St. Louis, MO, USA). To
127 generate astrocytes, cultures were maintained at 37 °C in an atmosphere of 5 % CO₂
128 for 7 days until confluence was achieved. Confluent cells were passaged to generate
129 purified astrocyte cultures. Microglia were isolated at day 13 *in vitro* by shaking for 45-
130 60 minutes and plated on adherent dishes supplemented with DMEM-F12 with 10%
131 FBS and maintained for more 24 hours. Hippocampal neurons were obtained from 16-
132 day embryonic mice and maintained in Neurobasal medium (Invitrogen; Carlsbad, CA,
133 USA) supplemented with B-27 (Life Technologies, Carlsbad CA, USA), penicillin,
134 streptomycin (0.5 mg/mL, HyClone), glutamine (2×10^{-3} M), and fungizone (0.65 μ M,
135 Sigma Aldrich, St. Louis, MO, USA). Cultures were maintained at 37°C in a humidified
136 atmosphere with 5% CO₂ at 9 days *in vitro*. Cell homogenates were collected,
137 centrifuged at 10000 g for 5 min at 4°C, and used for LXA4 measurements.

138

139 **Brain organoids**

140 Brain organoids were generated from GM23279A induced pluripotent stem cell (iPSC)
141 line as previously described²⁷. Briefly, 9,000 iPSC per well were plated of an ultra-low
142 attachment 96-well plate in hESC medium (20% knockout serum replacement; Life
143 Technologies) containing 50 μ M ROCKi (Y27632; Merck Millipore, USA) and 4 ng/ml b-
144 FGF. The plate was spun, and cells kept for 7 days to allow formation of embryoid
145 bodies (EBs). On the seven following days, EBs were changed to ultra-low attachment

146 plates, submitted to a Matrigel bath for 1h and media changed from neuroinduction
147 media [1% N2 supplement (Gibco), 1% GlutaMAX (Life Technologies), 1% MEM-
148 NEAAs, 1% P/S, and 1 µg/ml heparin in DMEM/F12 (Life Technologies)] to
149 differentiation media minus vitamin A (50% neurobasal medium, 0.5% N2, 1% B27
150 supplement without vitamin A, 1:100 2-mercapto-ethanol, 0.5% MEM-NEAA, 1%
151 GlutaMAX, and 1:100 P/S in DMEM/F12). On day 15, organoids were transferred to
152 agitation in 6-well plates at 90 rpm, and media was changed to differentiation media
153 plus vitamin A (50% neurobasal medium, 0.5% N2, 1% B27 supplement with vitamin A,
154 1:100 2-mercapto-ethanol, 0.5% MEM-NEAA, 1% GlutaMAX, and 1:100 P/S in
155 DMEM/F12), and replaced every four days until ready for experiment. On day 45, brain
156 organoids were fixed overnight in 4% PFA, then dehydrated in 30% sucrose, frozen in
157 optimal cutting temperature compound on dry ice, and sectioned (20 µm thickness) with
158 a cryostat (Leica Biosystems, Germany).

159

160 **Mouse strains**

161 Swiss albino mice were provided by Fundação Oswaldo Cruz (FIOCRUZ); inbred
162 C57BL/6 were provided by Charles River and 5-LOX knockouts (and wild-type
163 littermates) were provided by FIOCRUZ and kept in the animal facilities at the Federal
164 University of Rio de Janeiro. Adult male mice were used throughout the study and
165 tested during the light phase of the light cycle. Mice were maintained on a 12 h
166 light/dark cycle with food and water *ad libitum*, with maximum of 5 mice per cage.

167

168 ***In vivo* treatments**

169 Adult male Swiss mice received an intraperitoneal (i.p.) injection of 0.3 mg/kg
170 lipopolysaccharide (LPS) or vehicle immediately after contextual fear conditioning
171 training session. One hour later, mice received an intracerebroventricular (i.c.v.)
172 injection of either LXA4 (1 pmol) or vehicle and were tested for memory 7 days later.

173

174 **Immunofluorescence**

175 For immunofluorescence in brain organoids, frozen sections were rinsed with PBS and
176 permeabilized with PBS-Triton 0.3% (PBST) for 15 min. Slides were then incubated with
177 0.01M citrate buffer with 0.05% Tween 20 pH 6 for 10 min at 98°C for antigen retrieval
178 and blocked with blocking solution (PBS with 3% BSA) for 2 hours at room temperature.
179 Primary antibodies against 5-LOX (Cayman Chemical, 1:100) or MAP2 (Sigma-Aldrich,
180 1:300) diluted in blocking solution were incubated at 4°C overnight. Sections were then
181 rinsed with PBS and incubated in AlexaFluor-conjugated IgG secondary antibody goat
182 anti-mouse, rabbit or goat (Invitrogen, 1:400) for 1 hour at room temperature and
183 washed 3 times for 5 min in PBS. For nuclear staining, sections were incubated with
184 DAPI for 5 min. Slides were washed again 3 times for 5 min in PBS and then cover-
185 slipped with Aqua-Poly/Mount (Polysciences, Inc). Images were acquired using a Leica
186 TCS SP8 confocal microscope. For immunofluorescence experiments in the mouse
187 hippocampus, mice were transcardially perfused with saline and 4% formaldehyde.
188 Brains were removed and post-fixed in 4% formaldehyde overnight, then submerged in
189 30% sucrose solution. Forty μm -thick hippocampal cryosections were obtained,
190 mounted on glass slides, and exposed to 0.01M citrate buffer for antigen retrieval for 20
191 min at 60°C. Tissue was permeabilized with 0.5% Triton X-100 in 50 mM ammonium

192 chloride for 20 min at room temperature. Sections were then incubated in blocking
193 solution (5% bovine serum albumin in PBST) for 60 min at room temperature and then
194 incubated with anti-CB1R (Merck Millipore, 1:1000) in blocking solution overnight at 4
195 °C. Sections were then rinsed with PBST and incubated with AlexaFluor-conjugated
196 anti-rabbit IgG secondary antibodies (1:400) for 2 hours at room temperature, followed
197 by a short 5 min incubation in a DAPI solution (Molecular Probes). A 5 min incubation in
198 1% Sudan Black B prepared in 70% ethanol was applied to quench autofluorescence.
199 Tissue was mounted on coverslips with Aqua Poly/Mount (Polysciences) and imaged on
200 a Zeiss AxioImager M2 microscope. Image fluorescence was quantified on ImageJ²⁸
201 after selection of the stratum pyramidale and stratum radiatum of hippocampal CA1 and
202 CA3 subfields as regions of interest.

203

204 **Human samples**

205 CSF samples for this study were obtained from a cohort recruited at the D'Or Institute of
206 Research and Education (IDOR) in Rio de Janeiro, Brazil. This cohort comprised
207 healthy controls (HC; N = 25), and cognitively impaired subjects with diagnosis of either
208 amnesic mild cognitive impairment (aMCI; N = 13), Alzheimer's disease (N = 14), or
209 dementia with Lewy bodies (DLB; N = 9). Inclusion criteria for this study were: age > 60
210 years; absence of other neurological conditions, neurodevelopmental or genetic
211 diseases; native Brazilian Portuguese speakers; formal education \geq 8 years; no
212 restriction for MRI studies; no severe metabolic disease). All patients were evaluated
213 with the same extensive clinical, neuropsychological and neuroimaging investigation as
214 described²⁹⁻³³. For demographics and biomarker information, see Table 1. CSF

215 samples were collected by lumbar puncture performed around 11 a.m. in all cases, to
216 minimize circadian fluctuations. CSF was centrifuged, and the supernatant was
217 collected, aliquoted, and immediately frozen at -80°C . Before assays, samples were
218 thawed and kept on ice until use. Samples and calibrators were run in duplicates.

219

220 **ELISA**

221 Mouse brains were harvested, and lipid extraction was performed with ethanol (5 $\mu\text{L}/\text{mg}$
222 of wet tissue) followed by centrifugation for 5 min at 10,000 \times g. The supernatant was
223 applied directly into a double-sandwich ELISA kit, read at 650 nm and normalized by
224 wet tissue weight (g). Plasma and CSF samples and culture supernatants were applied
225 to the ELISA kits according to manufacturer's instructions. ELISA kits for LXA4 (#EA46)
226 were from Oxford Biomedical Research (Rochester Hills, MI). ELISA kits for $\text{A}\beta_{42}$, and
227 total tau (t-tau) were provided by Euroimmun (Lübeck, Germany) and experiments
228 were run following manufacturer's instructions.

229

230 **Inhibitory avoidance**

231 The step-down inhibitory avoidance apparatus consisted of a box measuring 26 x 10 x
232 35 cm with a 10 x 10 x 4 cm platform placed in the center, surrounded by a floor made
233 of parallel bronze bars and connected to a power source. For the training sessions,
234 mice were placed on the platform and when they stepped down with four paws onto the
235 grid they received a 0.7 mA foot shock for 2 s, and immediately returned to their home
236 cage. For the test session (30 min or 24 h after training), animals were again placed on
237 top of the platform, and latency to step down was recorded. For the protocol with

238 multiple trials, a milder shock of 0.5 mA was used, and the procedure was repeated until
239 the animal learned to remain 180 s on the platform for 180 s (criteria). For memory
240 extinction, mice were conditioned in the step-down inhibitory avoidance task, as
241 described above, only with a 1 mA foot shock intensity. Starting on the 12th day after
242 conditioning, mice were submitted to successive extinction trials in 24 h intervals, where
243 they were placed on the platform and allowed to step down onto the grid in the absence
244 of a foot shock. The latency to step down was recorded and mice were allowed to freely
245 explore the apparatus for 30 s after every trial. Reduction in step-down latency across
246 successive trials indicated extinction learning.

247

248 **Contextual Fear Conditioning**

249 To assess contextual fear memory consolidation, a two-phase protocol was used. Swiss
250 mice were initially trained in the conditioning cage (40 x 25 x 30 cm) and were allowed
251 to freely explore for 3 minutes followed by application of a single foot shock (1 mA) for 2
252 s. Mice were kept for another 1 minute in the cage and removed. Right after training,
253 mice received an intraperitoneal injection of 0.3 mg/kg lipopolysaccharide (LPS)
254 obtained from *E. coli* 0127:B8 (Sigma-Aldrich) or vehicle. One hour later, mice received
255 an intracerebroventricular (i.c.v.) injection of either LXA4 (1 pmol) or vehicle. Seven
256 days after training, mice were presented to the same cage for 5 minutes without
257 receiving a foot shock. Freezing behavior was recorded automatically using the
258 Freezing software (Panlab; Cornella, Spain). In all behavioral experiments, the
259 experimenter was blinded to the groups tested.

260

261 **Statistical analysis**

262 Statistical analyses were performed using GraphPad Prism 6 software (La Jolla, CA) or
263 the IBM SPSS Statistics v. 26 (Armonk, NY). Differences between two independent
264 groups were analyzed using Student's t-test. When three or more independent
265 experimental groups were compared, one- or two-way ANOVA was used, followed by
266 appropriate *post hoc* tests, as stated in "Figure Legends". For correlations, data
267 distribution was initially assessed using Shapiro-Wilk Test. After adjustment for age,
268 partial rank correlations were performed to assess the relationship between CSF LXA4
269 levels and clinical/biomarker variables. Significance level was set at 0.05. The predictive
270 power of CSF LXA4, alone or in combination with A β ₄₂, to identify clinical AD was tested
271 by plotting receiver operating characteristic (ROC) curves, widely used to determine the
272 diagnostic potential of biomarkers, and determining the area under the curve (AUC) with
273 a confidence interval of 0.95.

274

275 **Results**

276 *LXA4 is produced by neurons and microglia*

277 Lipoxins, including LXA4, are locally produced by immune cells at sites of
278 inflammation or systemically ¹¹. Although we and others have previously reported that
279 LXA4 is bioactive in the brain ^{14, 15, 19}, whether brain cells comprise a source for LXA4
280 remains unknown. We first used primary cultures to investigate whether brain cells
281 produced LXA4 at detectable levels. We found that mouse hippocampal neurons and
282 microglia had significantly higher content of LXA4 than astrocytes (Fig. 1a). We next
283 used human induced pluripotent stem cells (iPSCs) to derive human neural progenitor

284 cell (NPC), neuron, astrocyte or microglial-like cultures to determine the levels of LXA4.
285 We found that human iPSC-derived neurons produce considerably higher levels of
286 LXA4 than astrocytes or NPCs (Fig. 1a). Furthermore, in line with mouse results, levels
287 of human microglia-sourced LXA4 are comparable to human neurons, suggesting
288 microglia as a key producer of LXA4 (Fig. 1a). To further ascertain whether neuronal 5-
289 LOX expression would be present in more complex systems, we performed
290 immunohistochemical experiments to label 5-LOX in 45-day-old human brain organoids
291 derived from iPSC. At that stage, human brain organoids express a biochemical profile,
292 patterning, and structural organization similar to what is observed in the human
293 embryonic brain ²⁷. We observed prominent 5-LOX labeling in microtubule-associated
294 protein 2 (MAP2)-positive cells (Fig. 1b), confirming that human neurons typically
295 located in the outer layers of human brain organoids express 5-LOX. Together, our
296 results raise the possibility that neurons and microglia are important sources of LXA4 in
297 the human brain.

298

299 *Aging reduces systemic and brain LXA4 levels in mice*

300 Since aging impairs systemic response to injuries ^{34, 35} and renders the brain
301 vulnerable to neurodegenerative conditions ³⁶, we next determined whether levels of
302 LXA4 would be modified by aging in mice. We found that plasma and brain LXA4 levels
303 were reduced in 12-month-old male Swiss mice compared to young 3-month-old
304 controls (Fig. 2a,b). This is accompanied by evident short-term memory impairment in
305 the inhibitory avoidance memory test, as aged mice presented reduced latency to step

306 down from the platform in the test sessions (Fig. 2c). Results indicate that reduced brain
307 and peripheral LXA4 levels concur with cognitive deficits in aged mice.

308

309 *Suppression of LXA4 mimics age-associated memory loss in mice*

310 We next hypothesized that reductions in LXA4 would be causally implicated in
311 memory impairments in mice. Therefore, we tested memory performance in 3-month-old
312 (young) 5-LOX homozygous knockout mice (5-LOX^{-/-}), which show reduced circulating
313 LXA4 levels¹⁹, and their respective wild-type littermates (WT) in the inhibitory
314 avoidance memory task. We used adult 5-LOX^{-/-} mice in these experiments to mimic
315 reductions in circulating LXA4 and specifically dissect the roles of 5-LOX, while avoiding
316 potential confounders triggered by normal aging. Control experiments revealed no
317 differences in body weight (Supp. Fig. S1a) or food and water intake (Supp. Fig. 1b,c)
318 across genotypes. Additionally, levels of hippocampal CB1 receptors were similar in 5-
319 LOX^{-/-} and WT mice (Supp. Fig. 1d-f).

320 5-LOX^{-/-} mice took significantly more learning sessions in the inhibitory avoidance
321 task to reach criteria than WT (Fig. 2d). While WT showed normal memory
322 performance, 5-LOX^{-/-} mice presented impaired short-term, but not long-term memory
323 (Fig. 2e,f). Furthermore, 5-LOX^{-/-} mice had impaired extinction of learned fear memory
324 (Fig. 2g), which is in further agreement with learning deficits. Altogether, these results
325 demonstrate that 5-LOX-mediated LXA4 synthesis supports proper cognitive function
326 and indicate that reductions in LXA4 may render the brain vulnerable to dysfunction and
327 memory impairment.

328

329 *Administration of LXA4 attenuates inflammatory responses and memory loss*

330 Our results so far indicated that LXA4-mediated signaling could be part of a
331 protective mechanism against age-related cognitive decline, which is, at least in part,
332 mediated by inflammation³⁷. We then sought to determine whether administration of
333 LXA4 would protect against impairments in memory consolidation induced by an
334 inflammatory injury. Mice were trained in a contextual fear conditioning paradigm and
335 received a post-training intraperitoneal (i.p.) injection of 0.3 mg/kg lipopolysaccharide
336 (LPS) or vehicle. One hour after LPS, mice received an intracerebroventricular (i.c.v.)
337 injection of either LXA4 (1 pmol) or vehicle and were tested for memory 7 days later.
338 We found that i.c.v. LXA4 administration rescued impaired contextual fear memory
339 consolidation in LPS-injected mice (Fig. 3a), advocating towards a neuroprotective
340 effect via reduction of inflammatory pathways.

341 We thus attempted to determine the impact of LXA4 on LPS-induced
342 inflammatory cytokine production. I.c.v. LXA4 administration reduced levels of
343 interleukin 1 β (IL-1 β) (Fig. 3b,c) and interleukin 6 (IL-6) (Fig. 3d,e) in the brain and
344 plasma of LPS-treated mice. These results indicate that LXA4 attenuates peripheral and
345 brain inflammation, and rescues inflammation-induced cognitive defects in mice.

346

347 *Cerebrospinal fluid LXA4 declines with aging and dementia in humans*

348 To test the potential relevance of LXA4 in humans, we investigated LXA4 levels
349 in the cerebrospinal fluid (CSF) of a cohort of human subjects in a memory clinic. They
350 were diagnosed as mild cognitive impairment (MCI), AD, dementia with Lewy bodies
351 (DLB), or controls (see Supp. Table 1 for demographics and biomarker information). We

352 initially found that LXA4 declines with aging in human CSF in this cohort (Fig. 4a), in line
353 with our mouse studies. We further determined that LXA4 levels were markedly
354 decreased in subjects with AD or DLB, but not in MCI, as compared to controls (Fig.
355 4b). Together, these results support the notion that aging leads to a reduction in brain
356 LXA4 levels. The decrease in LXA4-mediated protective mechanisms may lead to
357 vulnerability to cognitive diseases and, presumably, neurodegeneration.

358

359 *Cerebrospinal fluid LXA4 correlates with memory performance, BDNF, and A β ₄₂ levels*
360 *in humans*

361 We then investigated whether CSF LXA4 would associate with memory
362 performance and the levels of markers relevant to AD. CSF LXA4 showed positive
363 correlations with mini-mental state exam (MMSE) scores, a proxy for memory
364 performance in humans (Fig. 4c). We further found that LXA4 positively correlates with
365 BDNF, a neurotrophin essential for memory function^{38, 39}, in the CSF (Fig. 4d). These
366 results indicate that central LXA4 actions may favor brain homeostasis and cognition.

367 We next addressed potential associations between CSF LXA4 and AD
368 biomarkers (A β ₄₂ and tau). We found that CSF LXA4 positively associates with CSF
369 A β ₄₂ (Fig. 4e), but not with tau (Supp. Fig. 2). Finally, combination of CSF LXA4 and
370 A β ₄₂ slightly increased the sensitivity and specificity of AD diagnostics in a receiver
371 operating characteristic (ROC) curve (Fig. 4f). These results suggest that declines in
372 CSF LXA4 correlate with brain A β ₄₂ accumulation, possibly facilitating its neurotoxic
373 actions.

374

375 **Discussion**

376 Age-related cognitive impairment and dementia are amongst the causes of
377 significant disability in the elderly and effective interventions are still not available.
378 Nonetheless, it is well established that loss of immune homeostasis and aberrant
379 inflammatory responses comprise significant factors predisposing individuals to brain
380 dysfunction and dementia^{40, 41}. Here we provide evidence suggesting that LXA4 is part
381 of an endogenous protective mechanism that decreases with aging and renders the
382 brain vulnerable to memory failure and dementia.

383 We first addressed a long-standing question of whether brain cells produce
384 LXA4. Previous evidence indicated that rodent neurons and microglia express 5-LOX,
385 the enzyme required for LXA4 synthesis^{16, 17}. Nonetheless, direct evidence for LXA4
386 production in brain cells was lacking. We found that mouse and human neurons and
387 microglia in culture actively produce and release LXA4, whereas astrocytes produce
388 smaller amounts of LXA4. These results suggest that neurons and microglia could act
389 as local sources of LXA4 in the brain and builds upon our previous data showing that
390 LXA4 is present in the brain despite the absence of its canonical ALX receptor, at least
391 under physiological conditions¹⁹.

392 We and others have provided initial evidence suggesting that peripheral LXA4
393 levels are reduced with aging in mice and humans. While we have previously shown
394 that 12-month-old mice present reduced plasma levels of LXA4 as compared to 3-
395 month-old mice¹⁴, Gangemi et al. (2005) demonstrated that urinary LXA4 is markedly
396 reduced in aged humans³⁴. We now confirmed these previous results in plasma and

397 extended our studies to demonstrate that central LXA4 also declines with age and
398 accompanies memory impairment in mice.

399 To further support that reduced LXA4 underlies memory impairment, we
400 determined that 5-LOX^{-/-} mice, which present reduced plasma and brain LXA4 content,
401 present learning impairment in an inhibitory avoidance task. We decided to use 3-
402 month-old 5-LOX^{-/-} mice (instead of aged subjects) in these experiments to circumvent a
403 potential source of confusion with other aspects related to the aging process. For
404 instance, 5-LOX^{-/-} mice have been reported to develop additional age-dependent
405 behavioral alterations, such as anxiety-like behavior⁴², which are not present in young
406 mice¹⁴. Together, these results demonstrate that reduced LXA4 is sufficient to trigger
407 memory failure in mice. Future studies are warranted to determine the relative
408 contributions of circulating and brain LXA4 to cognition by ablating 5-LOX in specific
409 sources of LXA4 (i.e. neurons, microglia, neutrophil, platelets).

410 5-LOX was previously shown to have a deleterious impact in mouse models of
411 AD and tauopathy⁴³⁻⁴⁶, potentially due to dyshomeostasis of other lipid derivatives.
412 Conversely, stimulation of LXA4 signaling through aspirin-triggered lipoxin (ATL)
413 resulted in beneficial actions, including reduced microglial reactivity, less A β ₄₂
414 accumulation and tau phosphorylation, and attenuation of memory impairment in mouse
415 models of AD^{15, 20}. Notably, LXA4 was reported to attenuate A β -induced memory
416 impairment through CB1 receptors¹⁹. Our findings further demonstrate that brain
417 administration of LXA4 prevents inflammation-induced cytokine upregulation and
418 memory consolidation defects in mice. Results suggest that preserving LXA4 signaling

419 across adulthood might be beneficial to ward off persistent inflammation, cognitive
420 decline, and AD risk at later stages of life.

421 We have previously shown that LXA4 acts as an allosteric agonist of CB1
422 receptors in the brain, enhancing the affinity of CB1 receptors to anandamide¹⁹. Given
423 that endocannabinoid signaling through CB1 receptors is essential for proper brain
424 functions⁴⁷⁻⁵⁰, locally produced LXA4 could serve as a means of intercellular
425 communication in the CNS to fuel synaptic plasticity, potentiate astrocyte metabolism
426 and microglial surveillance. Conversely, reduced brain LXA4 could explain, at least in
427 part, compromised CB1 receptor signaling, which may then translate into memory
428 impairment⁵¹.

429 Our results show that CSF LXA4 is considerably reduced in patients with
430 dementia (AD and DLB), conferring translational relevance to the rodent studies.
431 Significant correlations of CSF LXA4 with BDNF and memory function in humans also
432 indicate potential pro-mnemonic actions of central LXA4 and highlight the need for
433 further translational investigation of the underlying mechanisms. An intriguing
434 observation relies on the positive correlation of CSF LXA4 with A β ₄₂, but not with tau, in
435 humans. This suggests that brain A β accumulation (as assessed by lower CSF A β ₄₂)
436 may moderate LXA4 levels in the CNS independently of tau pathology (as determined
437 by CSF tau). These results encourage additional investigation to clarify the interrelation
438 of A β ₄₂, tau and LXA4, as well as to determine whether incorporation of LXA4
439 measurements into a CSF biomarker panel can aid in the discrimination of a subset of
440 AD cases, thereby improving overall diagnostics.

441 Activation of the innate immune system appears to mediate cognitive decline and
442 AD pathogenesis^{37, 52}. Indeed, aberrant cytokine release^{53, 54} and abnormal innate
443 immunity response to brain A β deposition have been reported in mouse models and
444 patients of AD^{55, 56}. Infiltration of immune cells, such as neutrophils⁵⁷, into the brain
445 may shift cellular responses towards reduced LXA4 production and, consequently,
446 increased neurotoxicity in AD.

447 We hypothesize that LXA4 deploys a dual mechanism as an anti-inflammatory
448 and neuroprotective agent, thereby contributing to resilience of central nervous system
449 against disease-associated insults, such as A β_{42} in AD. Hence, LXA4 levels may be
450 useful as a biomarker of brain vulnerability, and its decreased levels may indicate a
451 homeostatic breakdown. Since a significant part of the CB1 receptor-related effects of
452 anandamide are due to synergistic LXA4 interaction¹⁹, the vulnerability window caused
453 by age-associated LXA4 reduction might be conceived as part of the “endocannabinoid
454 deficiency syndrome” hypothesis⁵⁸. This concept advocates that under circumstances
455 of relatively low endocannabinoid signaling, certain inflammation-linked injuries, such as
456 fibromyalgia, inflammatory bowel syndrome and migraine, become more incident. Here
457 we propose to extend this concept to include neurodegeneration and dementia.

458 In summary, our findings support the notion that LXA4 signaling may comprise
459 an endogenous protective mechanism that renders the brain vulnerable to the
460 exacerbated inflammation and neurodegenerative factors (i.e. A β_{42} deposition).
461 Preventing declines in LXA4 levels may preserve endocannabinoid signaling, brain
462 homeostasis, and cognition, thereby contributing to reduced odds of dementia. Future
463 studies are needed to establish the intricate signaling pathways initiated by brain

464 actions of LXA4, as well as to test whether stimulation of LXA4 (or the endocannabinoid
465 system) is effective in delaying cognitive decline in aged humans.

466

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475

476 **Author contributions**

477 FAP, FTM designed the study. FAP, MVL, FCR, GV, CM, PFL, KK, IMO, LML, BP, FKS,
478 GC, CD, NA, BV, and CC performed research. FAP, MVL, BP, and FKS analyzed data.
479 FAP, MVL, CC, HCCFN, SKR, FAB, and FTM contributed reagents, materials, animals,
480 and analysis tools. FAP, MVL, STF, FAB, and FTM analyzed and discussed results.
481 FAP and MVL wrote the manuscript.

482

483 **Conflict of Interest**

484 The authors declare no conflict of interests.

485

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721 **Figure Legends**

722

723 **Figure 1. LXA4 is produced by neurons and microglia.** (a) Levels of lipoxin A4
724 (LXA4) in primary cultures enriched in astrocytes, microglia or neurons derived from
725 mice (left) or in human iPSC-derived astrocytes, microglia, neurons, or neural progenitor
726 cells (NPCs). Unpaired two-tailed one-way ANOVA with Šidák post hoc test (***p* <
727 0.001; *****p* < 0.0001; n.s. non-significant). n.d.; not detected. (b) Representative
728 images of immunofluorescence experiments (5-lipoxygenase, 5-LOX immunoreactivity:

729 green; microtubule-associated protein 2, MAP2 immunoreactivity: red; DAPI: blue; N=3)
730 in human iPSC-derived brain organoids. Scale bar: 10 μ m.

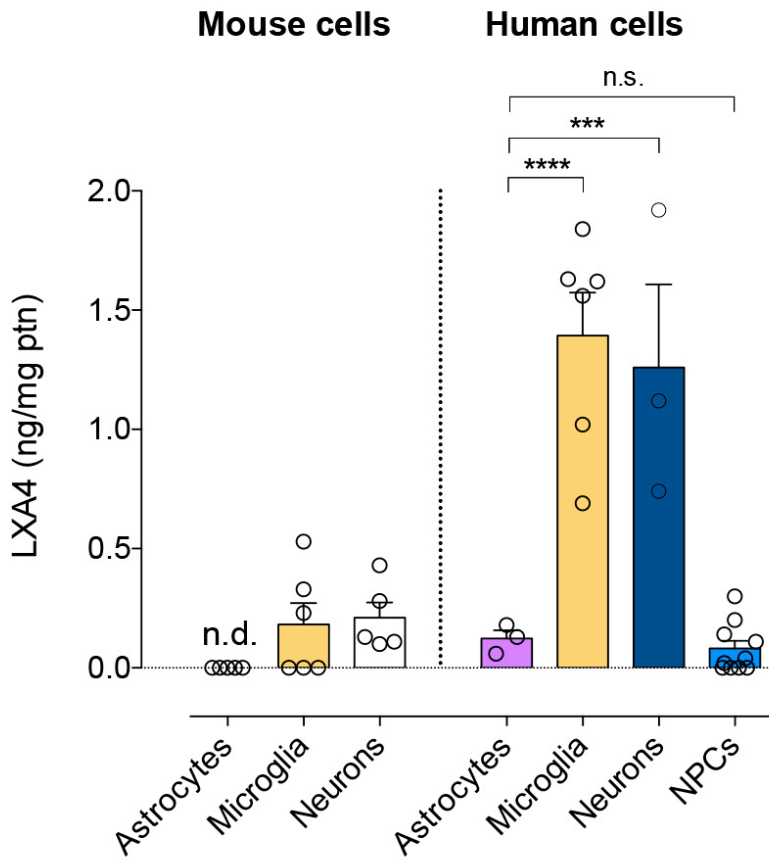
731
732 **Figure 2. Age-linked reductions in LXA4 result in cognitive impairment.** (a,b)
733 Levels of lipoxin A4 (LXA4) in plasma (a) and brain (b) of young (3 month-old) or aged
734 (12 month-old) Swiss mice. (c) Step-down latency of young or aged mice in the
735 inhibitory avoidance task. (d) Number of trials required for each mouse to reach the
736 criteria during the training session in the inhibitory avoidance fear task. (e,f) Step-down
737 latency of 5-LOX^{-/-} or WT mice (3 month-old) in the inhibitory avoidance task to assess
738 short-term (e; 1 hour after training) or long-term memory (f; 24 hours after training). For
739 a, b, and d, two-tailed unpaired Student's t-test. For c, e, and f, two-tailed unpaired
740 Mann-Whitney (* p < 0.05; **** p < 0.0001). Graphs show means \pm standard error of the
741 mean (SEM). Each dot represents an individual. (g) Fear memory extinction assessed
742 by step-down latency of 5-LOX^{-/-} or WT after repeated test sessions twelve to fifteen
743 days after original conditioning session. Repeated measures one-way ANOVA with
744 Šidák post hoc test (* p < 0.05). Graphs show means \pm SEM.

745
746 **Figure 3. LXA4 attenuates inflammation-induced memory failure and cytokine**
747 **production in mice.** (a) Freezing (seconds) of control or LPS-injected mice (0.3 mg/kg)
748 treated with vehicle or 1 pmol LXA4 (i.c.v) in the contextual fear conditioning task. (b-e)
749 Brain and plasma levels of IL-1 β (b,c) or IL-6 (d,e) in control or LPS-injected mice (0.3
750 mg/kg; i.p.) treated with vehicle or 1 pmol LXA4 (i.c.v). Two-tailed unpaired two-way
751 ANOVA followed by Holm-Šidák post hoc test (* p < 0.05; ** p < 0.01; *** p < 0.001; ****
752 p < 0.0001). Graphs show means \pm standard error of the mean (SEM).

753
754 **Figure 4. Cerebrospinal fluid LXA4 is reduced in aging and in dementia in**
755 **humans.** (a) Correlation between age (in years) and CSF LXA4 in human subjects. (b)
756 CSF levels of LXA4 in AD and DLB patients compared to healthy controls or amnesic
757 MCI patients (N = 25 controls, 13 aMCI, 14 AD, 9 LBD patients). Two-tailed unpaired
758 one-way ANOVA followed by Holm-Šidák post hoc test (** p < 0.01; ns, non-
759 significant). Graphs show means \pm standard error of the mean (SEM). (c-e) Correlations

760 between LXA4 and MMSE scores (c), CSF BDNF (d) or $A\beta_{42}$ (e) levels in human
761 subjects. Lines represent partial rank correlations (r and p-values as indicated in
762 graphs), adjusted for age, and the confidence interval is represented as gray shade. (f)
763 Receiver-operating characteristic curves for diagnostic based on $A\beta_{42}$ alone (red line) or
764 LXA4* $A\beta_{42}$ (blue line); confidence interval: 0.95; $p < 0.001$. HC: healthy controls, white
765 symbols; aMCI: amnesic mild cognitive impairment, grey symbols; AD: Alzheimer's
766 disease, black symbols; DLB: dementia with Lewy bodies, golden symbols); AUC: area
767 under curve.

a



b

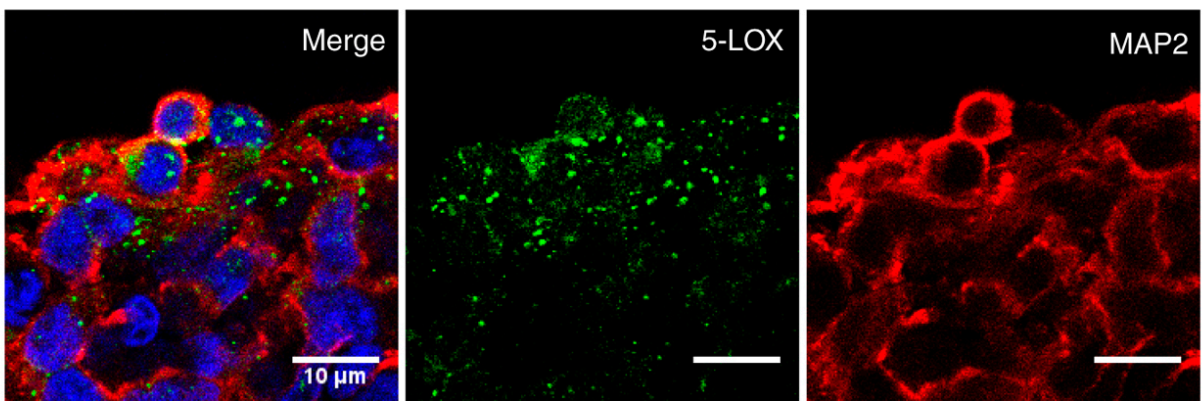


Figure 1

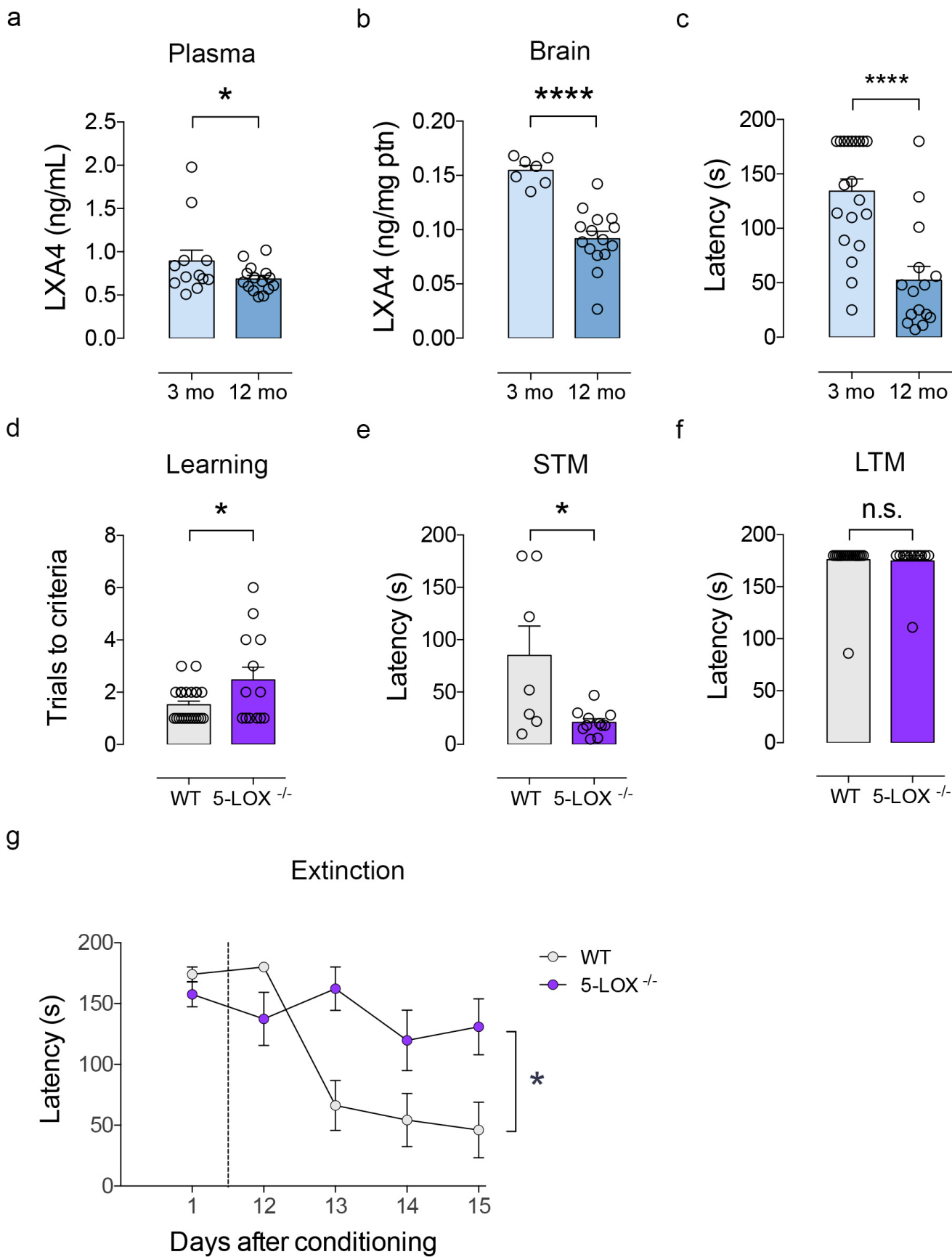


Figure 2

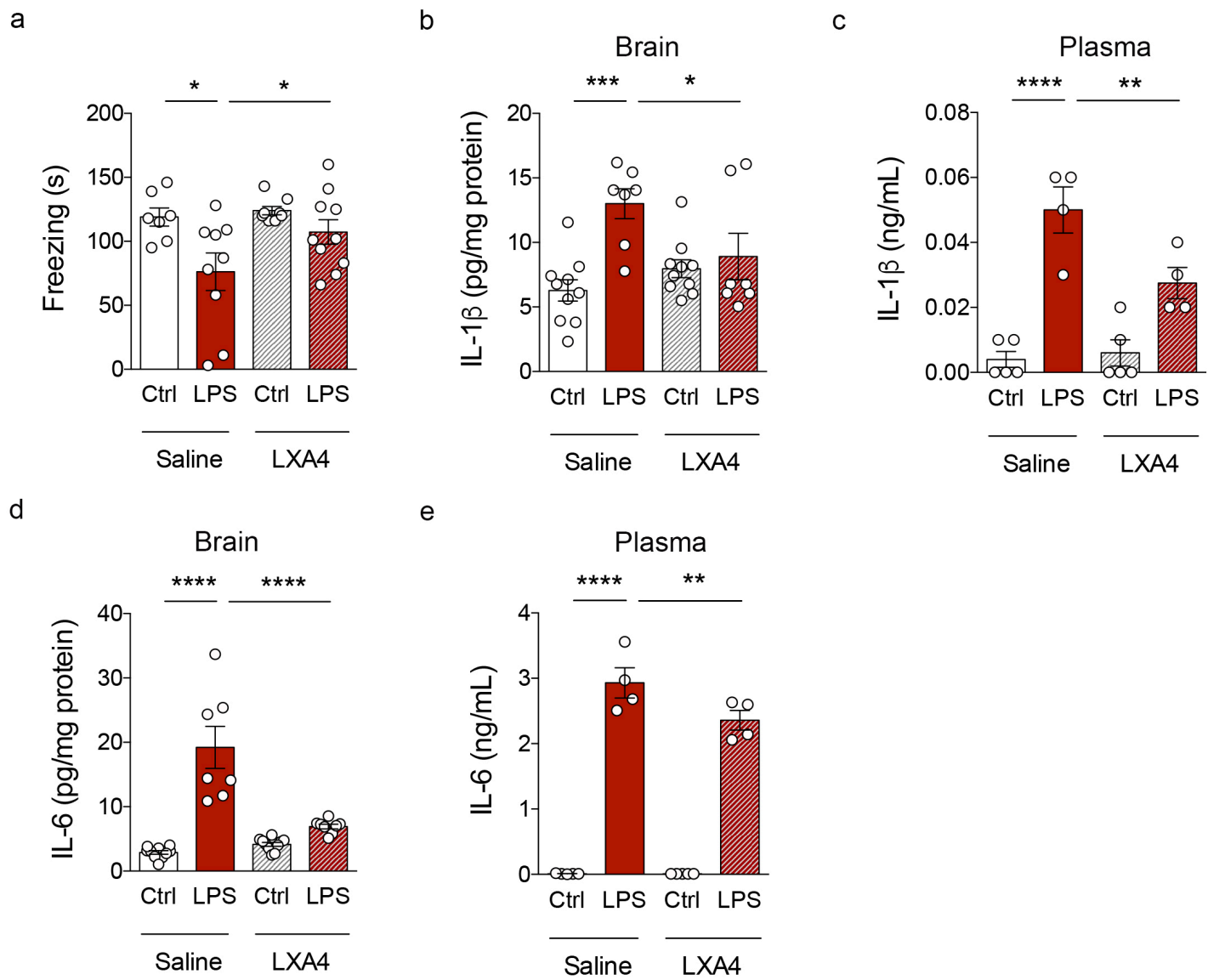


Figure 3

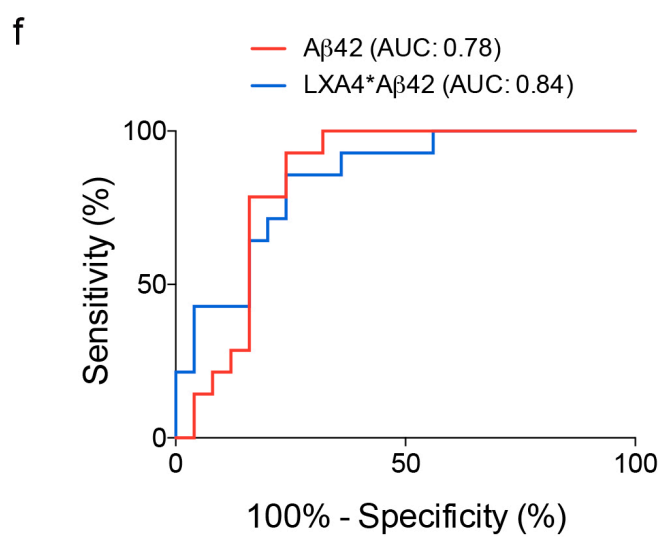
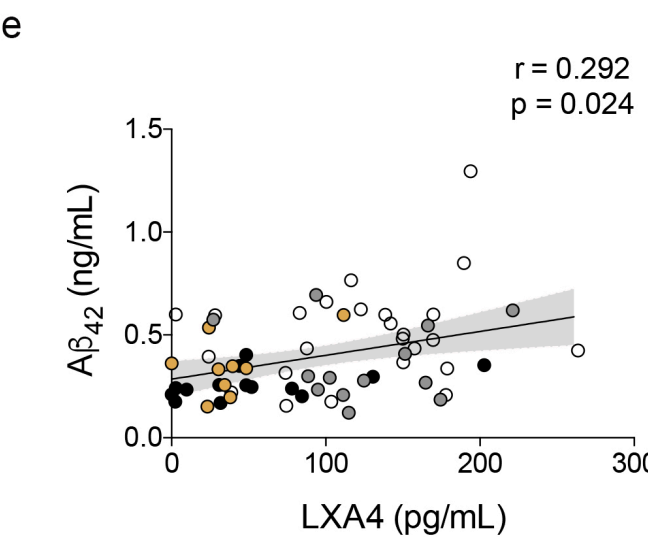
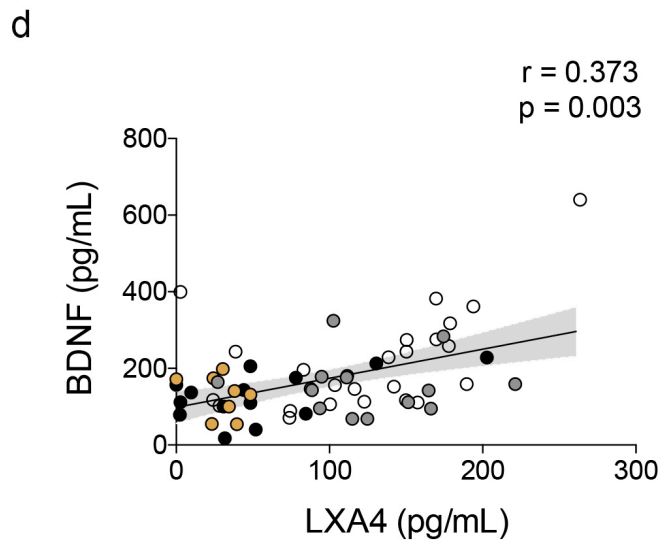
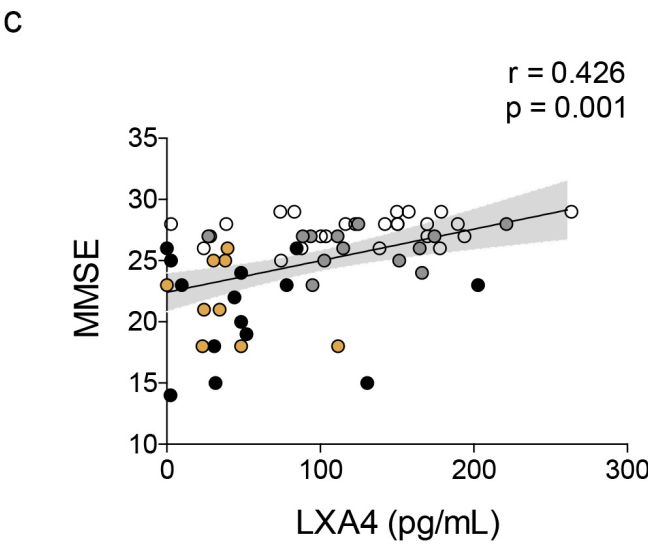
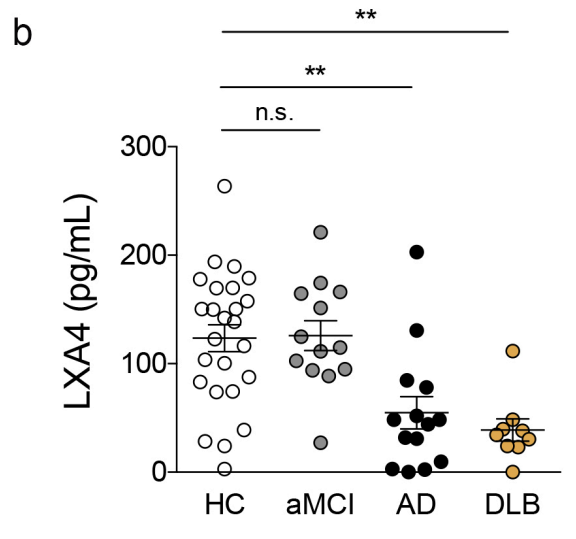
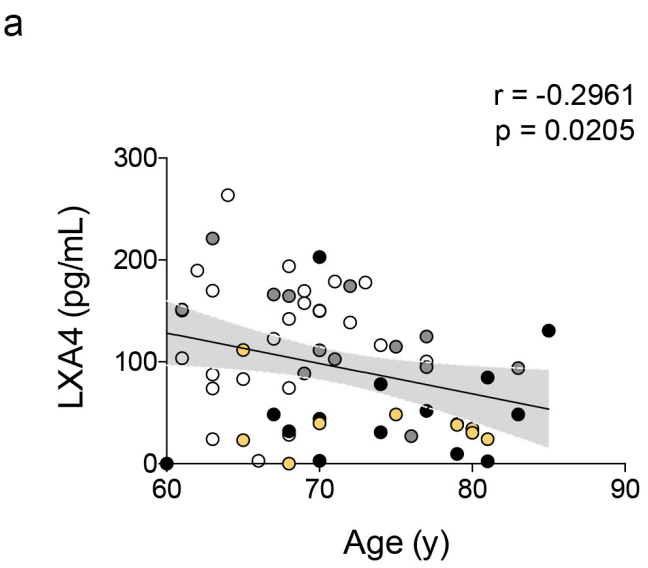


Figure 4