1   # Age-linked suppression of lipoxin A4 mediates cognitive deficits in

2   # mice and humans

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31

Abstract

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

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 (Aβ) 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

4

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, Aβ 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

5

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 days [23,

112   24]. 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

115   [25]. 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 described [26].

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 µM, Sigma Aldrich, St. Louis, MO, USA) and 10% fetal

125   bovine serum (FBS, Invitrogen; Invitrogen, Carlsbad, CA, USA). Cells were plated in 25

126   cm^2 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 µM,

135   Sigma Aldrich, St. Louis, MO, USA). Cultures were maintained at 37°C in a humidified

136   atmosphere with 5% CO^2 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 described [27]. 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 µM 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

7

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**

8

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

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

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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   ^{o}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 ImageJ [28]

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   amnestic 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 ≥ 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   described [29-33]. 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 perfomed with ethanol (5 µL/mg

222   of wet tissue) followed by centrifugation for 5 min at 10,000 × 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 $A\beta_{42}$, and

227   total tau (t-tau) were provided by Euroimmun (Lubeck, 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

11

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

12

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 $A\beta_{42}$, 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

14

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 levels [19], 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

15

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 inflammation [37]. 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 1β (IL-1β) (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

16

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 Aβ$_{42}$ 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 (Aβ$_{42}$ and tau). We found that CSF LXA4 positively associates with CSF

369   Aβ$_{42}$ (Fig. 4e), but not with tau (Supp. Fig. 2). Finally, combination of CSF LXA4 and

370   Aβ$_{42}$ 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 Aβ$_{42}$ accumulation, possibly facilitating its neurotoxic

373   actions.

374

Discussion

375

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 conditions [19].

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 mice [14], Gangemi et al. (2005) demonstrated that urinary LXA4 is markedly

396   reduced in aged humans [34]. We now confirmed these previous results in plasma and

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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 behavior [42], which are not present in young

406 mice [14]. 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 tauopathy [43-46], 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 $A\beta_{42}$

414 accumulation and tau phosphorylation, and attenuation of memory impairment in mouse

415 models of AD [15, 20]. Notably, LXA4 was reported to attenuate $A\beta$-induced memory

416 impairment through CB1 receptors [19]. 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

19

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 anandamide [19]. Given

423   that endocannabinoid signaling through CB1 receptors is essential for proper brain

424   functions [47-50], 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   impairment [51].

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 Aβ_{42}, but not with tau, in

435   humans. This suggests that brain Aβ accumulation (as assessed by lower CSF Aβ_{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 Aβ_{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.

20

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 Aβ deposition have been reported in mouse models and

444   patients of AD [55, 56]. Infiltration of immune cells, such as neutrophils [57], 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 $A\beta_{42}$ 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 interaction [19], the vulnerability window caused

453   by age-associated LXA4 reduction might be conceived as part of the "endocannabinoid

454   deficiency syndrome" hypothesis [58]. 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. $A\beta_{42}$ 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

21

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

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

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)

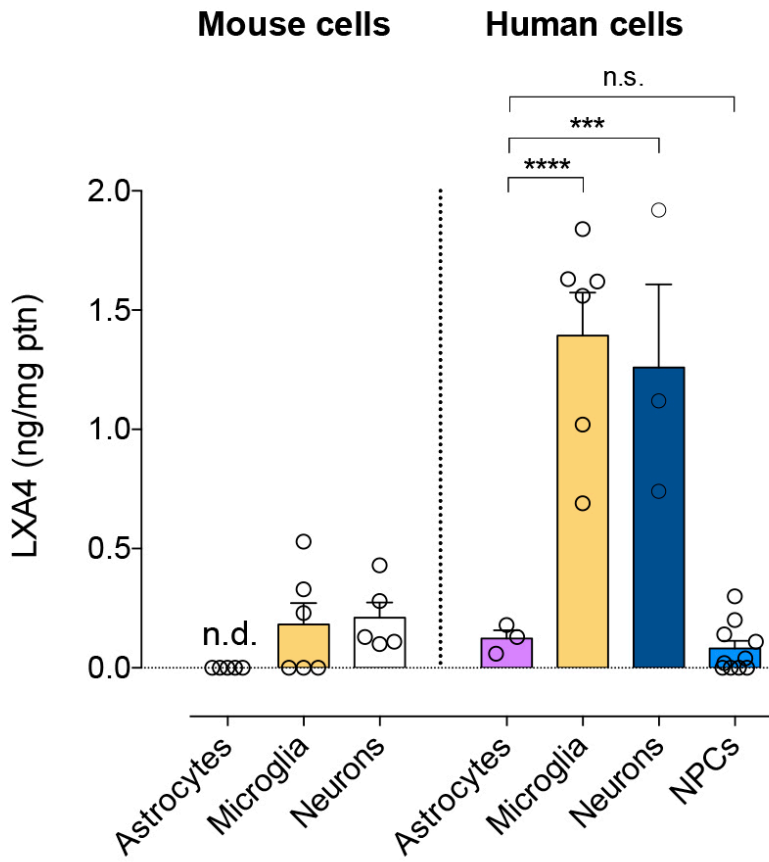730   in human iPSC-derived brain organoids. Scale bar: 10 μm.

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 ± 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 ± 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-1β (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 ± 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 amnestic

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 ± 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: amnestic mild cognitive impairment, grey symbols; AD: Alzheimer's

766   disease, black symbols; DLB: dementia with Lewy bodies, golden symbols); AUC: area
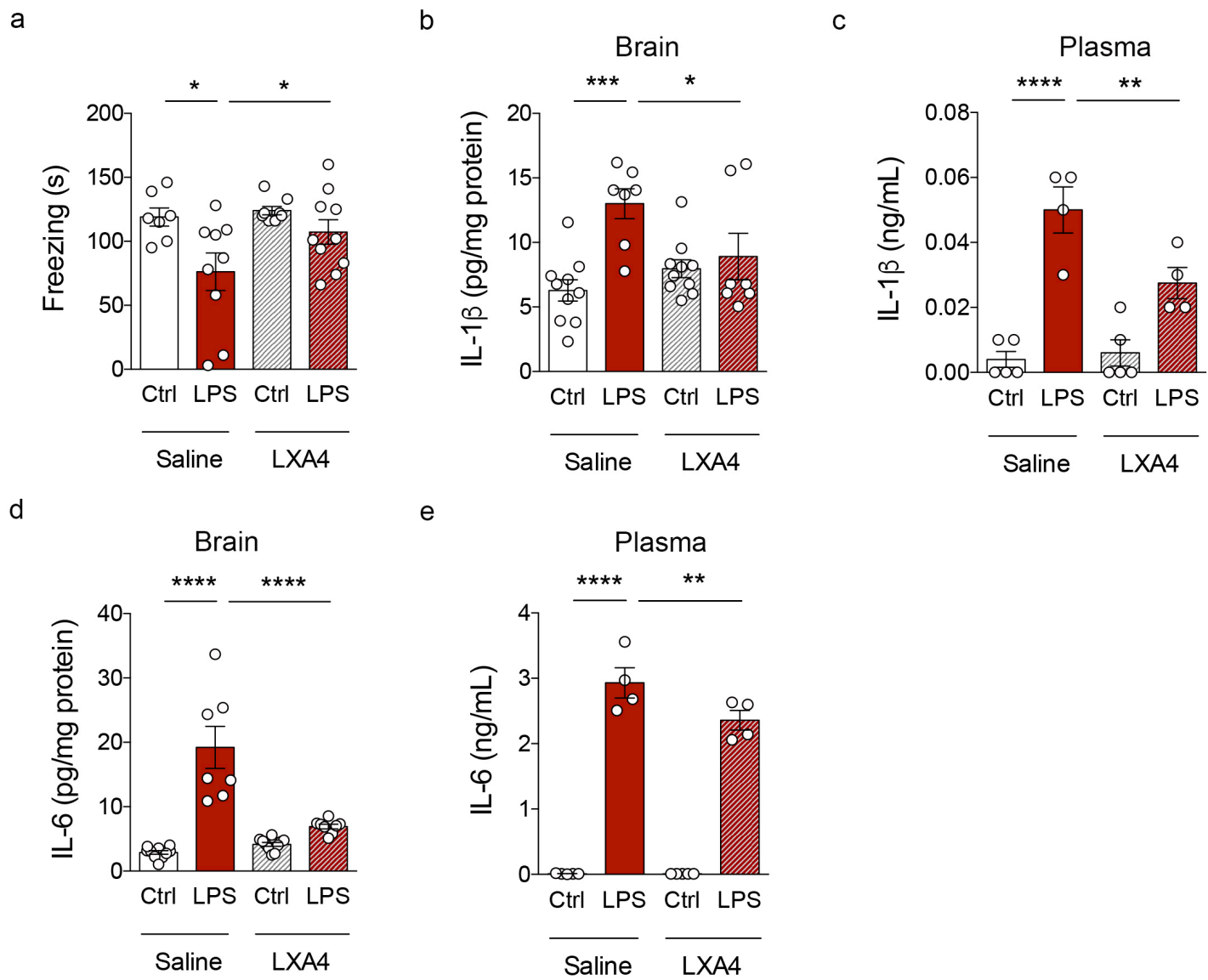
767   under curve.

a

Mouse cells   **Human cells**

b

Figure 1

a Plasma

b Brain

c

d Learning

e STM

f LTM

g Extinction

Figure 2

a

Freezing (s)

b Brain

IL-1β (pg/mg protein)

c Plasma

IL-1β (ng/mL)

d Brain

IL-6 (pg/mg protein)

e Plasma

IL-6 (ng/mL)

Figure 3

a

r = -0.2961
p = 0.0205

LXA4 (pg/mL) vs Age (y)

b

n.s.
**
**

LXA4 (pg/mL): HC aMCI AD DLB

c

r = 0.426
p = 0.001

MMSE vs LXA4 (pg/mL)

d

r = 0.373
p = 0.003

BDNF (pg/mL) vs LXA4 (pg/mL)

e

r = 0.292
p = 0.024

Aβ42 (ng/mL) vs LXA4 (pg/mL)

f

Aβ42 (AUC: 0.78)
LXA4*Aβ42 (AUC: 0.84)

Sensitivity (%) vs 100% - Specificity (%)

Figure 4