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1 **Astrocytic mitochondrial ROS modulate brain metabolism and mouse**
2 **behavior**

3

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34 Brain functioning is energetically a highly demanding process¹ that requires the
35 metabolic cooperation of different cell types²⁻⁴. Astrocytic-derived lactate sustains
36 memory consolidation⁵ by serving as an energetic oxidizable substrate for neurons⁶ and
37 a signaling molecule^{7,8}. Factors that support this metabolic compartmentalization are
38 differential regulation of glycolytic enzymes⁹ and mitochondrial respiratory chain
39 organization¹⁰ between neurons and astrocytes. Thus, unlike neurons, astrocytes rely
40 heavily on glycolysis for energy generation⁹ and, as a consequence, have a loosely
41 assembled mitochondrial respiratory chain that allows a higher generation of
42 mitochondrial reactive oxygen species (ROS)¹⁰. However, whether such a naturally
43 abundant mitochondrial ROS in astrocytes have any physiological function is unknown.
44 To address this issue, a genetically-engineered mouse was herein generated to
45 persistently lessen mitochondrial ROS in astrocytes during adulthood. Transcriptomics,
46 metabolomics, biochemical, immunohistochemical and behavioral scrutiny of these
47 mice revealed significant alterations in brain redox, carbohydrate, lipid and amino acid
48 metabolic pathways affecting neuronal function and mouse behavior. Notably, we found
49 that astrocytic mitochondrial ROS regulates glucose utilization *via* the pentose-
50 phosphate pathway and glutathione metabolism, through which they modulate the redox
51 status and survival of neurons. Our data thus indicate that astrocytic mitochondrial ROS
52 are physiological regulators of brain metabolism and neuronal function.

53

54 To down-regulate endogenous mitochondrial ROS abundance, we generated, by
55 homologous recombination in the *Rosa26 locus*, a transgenic mouse harboring the full-
56 length cDNA of catalase fused to the cytochrome *c* oxidase subunit VIII-mitochondrial
57 leading sequence, and to the human influenza hemagglutinin (HA) for tagging purposes
58 (mitochondrial-tagged catalase) (Figure 1a and Figure S1a-e). This construct has been
59 shown to be efficiently imported into mitochondria while maintaining its activity¹¹. A
60 floxed (LoxP-flanked) transcriptional STOP cassette was incorporated between the
61 mitochondrial-tagged catalase cDNA and the CAG promoter (mCAT^{LoxP/+}), in order to
62 eventually obtain tissue- and time-specific expression of mitochondrial-tagged catalase
63 *in vivo* (Figure 1a). mCAT^{LoxP/+} mice, bred under a C57BL/6-J background, was mated
64 with human cytomegalovirus minimal promoter (CMV)-Cre recombinase C57BL/6-J
65 mice (CMV-Cre/+). This led to a CMV-Cre/+; mCAT/+ mouse that was backcrossed
66 with C57BL/6-J mice to constitutive express mitochondrial-tagged catalase (mCAT/+
67 or mCAT mice) (Figure 1a). Astrocytes in primary culture obtained from mCAT or

68 littermate wild type (WT) mice, revealed efficient expression of catalase confined to
69 mitochondria, as judged by western blotting in whole cells (Figure 1b), subcellular
70 fractions (Figure 1c) and by immunofluorescence (Figure 1d). The efficacy of the
71 expressed mitochondrial-targeted catalase construct to down modulate mitochondrial
72 ROS was also validated, as judged by the ability of astrocytic isolated mitochondria to
73 remove exogenous H₂O₂ (Figure 1e), as well as to decrease endogenous mitochondrial
74 ROS (Figure 1f,g).

75

76 To achieve down-modulation of mitochondrial ROS in astrocytes *in vivo*, mCAT^{LoxP/+}
77 mice was mated with mice, under the C57BL/6-J background, harboring the estrogen
78 receptor (ERT2)-inducible (tamoxifen-dependent) Cre recombinase (Cre^{ERT2}) governed
79 by the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter (GFAP-
80 Cre^{ERT2/+})¹² (Figure 1a). The progeny, namely GFAP-Cre^{ERT2/+}; mCAT^{LoxP/+}, and +/+;
81 mCAT^{LoxP/+} littermates, at the age of 8 weeks were intraperitoneally injected with
82 tamoxifen to activate Cre recombinase activity in astrocytes *in vivo*. The resulting mice,
83 namely GFAP-Cre^{ERT2/+}; mCAT/+ (henceforth GFAP-mCAT) and +/+; mCAT^{LoxP/+}
84 littermates (henceforth Control) (Figure 1a) were analyzed during adulthood. To
85 confirm the occurrence of mitochondrial-tagged catalase in astrocytic mitochondria in
86 the adult GFAP-mCAT mice, whole brain cells were acutely dissociated, and astrocytes
87 purified using an immunomagnetic approach (Figure 1h). Effective expression of
88 mitochondrial-tagged catalase was confirmed by western blotting and immunostaining
89 (Figure 1i), and by H₂O₂ production assessment (Figure 1j) in the astrocytic fraction. To
90 further confirm the functional efficacy of mitochondrial catalase, we determined
91 mitochondrial ROS in astrocytes acutely dissociated from the GFAP-mCAT mouse
92 brain. For this, brain cells were isolated from mice previously intravenously injected,
93 through the retro-orbital sinus, with adeno-associated viruses (AAVs) expressing the
94 green fluorescence protein (GFP) under the control of the short GFAP promoter. Cells
95 were then subjected to mitochondrial ROS assessment by flow cytometry analysis using
96 MitoSox®, which revealed a 25% reduction in the GFAP-mCAT astrocytes (Figure 1k).
97 Thus, an experimental model was generated to investigate the biological consequences
98 of reducing naturally abundant mitochondrial ROS in astrocytes in cultured cells and *in*
99 *vivo*.

100

101 Next, we aimed to investigate whether reduction of mitochondrial ROS in adult
102 astrocytes altered the transcriptional profile *in vivo*. To do so, astrocytes were acutely
103 isolated from the brains of adult GFAP-mCAT and control mice by the
104 immunomagnetic approach and subjected to a whole-genome transcriptomics array
105 analysis. Data analysis (Figure S2a-h; Tables S1 and S2) revealed >600 significantly
106 altered transcripts in the GFAP-mCAT mice, including redox and energy metabolism
107 genes. To ascertain if the observed altered gene expression by mitochondrial ROS in
108 GFAP-mCAT astrocytes correlates with changes in metabolic pathways, we performed
109 an untargeted metabolomics study in the brain extracts of the GFAP-mCAT and control
110 mice. Data analysis (Figure S3a-g; Tables S3 and S4) revealed >50 significantly altered
111 metabolites in the GFAP-mCAT mice and, notably, increased ribose-5-phosphate/6-
112 phosphogluconate ratio and decreased pyruvate/glucose ratio, likely indicating
113 increased pentose-phosphate pathway (PPP) and decreased glycolysis. To confirm this
114 end, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and
115 western blotting in mCAT astrocytes were performed, which revealed increased
116 expression of G6PD (Figure 2a,b and Figure S4a), i.e. the first and rate-limiting enzyme
117 of the PPP¹³. G6PD is transcriptionally regulated¹⁴ by miR-206, the levels of which
118 were found decreased in the mCAT astrocytes, according to the RT-qPCR analysis
119 (Figure 2c). Interestingly, the ectopic expression of miR-206 in mCAT astrocytes
120 rescued the increase in G6PD mRNA (Figure 2d). Moreover, the nuclear abundance of
121 the redox sensor transcriptional modulator, histone deacetylase-4 (HDAC4), which
122 represses miR-206 expression¹⁵, was higher in mCAT astrocytes (Figure 2e and Figure
123 S4b) indicating the predominance of the reduced (active) form of HDAC4 in these
124 cells¹⁶. Furthermore, inhibition of HDAC activity prevented the increase in *G6pd*
125 mRNA abundance found in mCAT astrocytes (Figure 2f). These data suggest that, in
126 astrocytes, mROS normally keeps HDAC4 oxidized and localized in the cytosol thus
127 allowing miR-206 expression, which in turn destabilizes G6PD mRNA. Since G6PD
128 mRNA and/or protein abundances, alone, are not necessarily a reflection of G6PD-
129 mediated PPP activity in the intact cell, we next assessed the rate of ¹⁴CO₂ production
130 from mCAT astrocytes incubated in the presence of [1-¹⁴C]glucose, which is
131 decarboxylated in the PPP and in the tricarboxylic acid (TCA) cycle¹⁷. As shown in
132 Figure 2g (left panel), ¹⁴CO₂ production from [1-¹⁴C]glucose significantly increased in
133 mCAT when compared with control astrocytes; however, the rate of [6-¹⁴C]glucose
134 oxidation, which takes place exclusively in the TCA cycle¹⁷ was unaffected (Figure 2g,

135 middle panel). Thus, the difference between the rates of oxidation from [1-¹⁴C]- and [6-
136 ¹⁴C]glucose, which reflects the rate of glucose oxidation *via* PPP⁹, increased by 1.4-fold
137 in the mCAT astrocytes (Figure 2g, right panel). These data suggest that naturally
138 occurring mROS in astrocytes physiologically repress PPP activity. Notably, we
139 observed that mCAT astrocytes showed a decrease in the rate of glycolysis, as judged
140 by the decreased lactate release when compared with WT astrocytes (Figure 2h) and the
141 glycolytic conversion of [3-³H]glucose into ³H₂O (Figure 2i). These results are in good
142 agreement with the notion that PPP and glycolysis are mutually affected pathways in
143 several experimental paradigms⁹. Altogether, these data indicate that mROS
144 physiologically regulates glucose metabolism in astrocytes by repressing PPP and
145 promoting glycolysis.

146

147 Given the function of PPP to maintain the NADPH(H⁺)/NADP⁺ ratio^{1,13}, we then aimed
148 to ascertain whether mROS physiologically influences the redox status of this
149 nicotinamide dinucleotide. The NADPH(H⁺)/NADP⁺ ratio was significantly lower in
150 the mCAT when compared with WT astrocytes (Figure 3a), a result compatible with
151 increased NADPH(H⁺) consumption. NADPH(H⁺) oxidases (NOXs) are active
152 NADPH(H⁺) oxidizing enzymes¹⁸, and we previously reported that NOX-1 and -2 are
153 largely the most abundant NOXs isoforms present in astrocytes¹⁰. Interestingly, RT-
154 qPCR analysis of mCAT astrocytes revealed increased NOX-1 and -2 mRNA
155 abundances when compared with WT astrocytes (Figure 3b). Since these NOXs are
156 localized in the plasma membrane facing their active site to the extracellular space, we
157 next determined extracellular superoxide anion (O₂^{•-}) abundance. In good agreement
158 with the observed enhanced expression of NOX-1 and -2, we found higher extracellular
159 O₂^{•-} in mCAT when compared with WT astrocytes (Figure 3c). Since NOX-1 and -2
160 genes expression are regulated by the nuclear factor-erythroid 2-related factor-2
161 (NRF2)^{19,20}, we then analyzed NRF2 functional activity in mCAT astrocytes. As shown
162 in Figure 3d, the mRNA abundances of a battery of canonical NRF2-target genes were
163 decreased in mCAT astrocytes indicating reduced NRF2 activity when compared with
164 WT astrocytes. Given that ROS is known to stabilize NRF2, promoting its nuclear
165 translocation and activity²¹, we examined NRF2 subcellular distribution. As shown in
166 Figure 3e and Figure S4c-e, whilst total and cytosolic NRF2 protein abundances were
167 not altered, its nuclear abundance was lower in mCAT when compared with WT
168 astrocytes. Finally, we ascertained whether, by modulating the NRF2-NOXs pathway,

169 mROS could regulate extracellular $O_2^{\cdot-}$. Ectopic expression of a phosphomimetic (PM),
170 constitutively active form of NRF2 (NRF2-PM)²² in mCAT astrocytes rescued the
171 increased NOX-1 and -2 mRNA abundances (Figure 3f) along with extracellular $O_2^{\cdot-}$
172 release (Figure 3g). Together, these data indicate that, by promoting NRF2
173 transcriptional activity, mROS physiologically down modulates extracellular ROS
174 release from astrocytes.

175

176 Given that neurons metabolically depend upon astrocytes for normal function and
177 survival^{1,5,7}, we next investigated whether neurons were affected by the reduction of
178 mitochondrial ROS in astrocytes. Transcriptomics analysis of wild type neurons freshly
179 isolated from the GFAP-mCAT mice (Figure S4f) failed to identify statistically
180 significant differences in the transcriptome (Figure S4g,h). Whilst these data indicate
181 that astrocytic mROS do not significantly affect gene expression in neurons, this
182 approach may not reveal possible post-transcriptional modifications affecting neuronal
183 metabolism and redox status. To ascertain this and, in view that astrocyte supports
184 neuronal functions^{22,23}, we next aimed to ascertain whether astrocyte mROS regulate
185 aspects of astrocyte-neuronal redox communication. Amongst the NRF2-target genes
186 found to be down modulated in the mCAT astrocytes (Figure 3d), we focused on
187 glutamate-cysteine ligase, catalytic subunit (GCL), a key regulator of glutathione (GSH)
188 biosynthesis²⁴. GCL down modulation was validated by western blotting (Figure 3h and
189 Figure S4i), and its functional effect confirmed by determining GSH concentration,
190 which was lower in mCAT when compared with WT astrocytes (Figure 3i, left panel)
191 without altering the oxidized glutathione status (Figure 3i, right panel). Notably, both
192 the decrease in GCL protein abundance (Figure 3j and Figure S4j) and total GSH
193 concentration (Figure 3k, left panel) were rescued by the expression of the
194 constitutively active NRF2-PM; interestingly, this treatment increased the oxidized
195 glutathione status (Figure 3k, right panel). Next, we performed co-cultures of mCAT
196 astrocytes with wild type neurons (Figure 3l), which were analyzed after the removal of
197 astrocytes. As shown in Figure 3m, mCAT astrocytes promoted in neurons a decrease in
198 GSH concentration and in the oxidized glutathione status, whereas H_2O_2 abundance
199 increased. This suggests an enhanced GSSG conversion into GSH, likely as an attempt
200 to remove excess H_2O_2 , although cell damage occurred. Interestingly, these effects were
201 counteracted in neurons co-cultured with NRF2-PM-pretreated mCAT astrocytes
202 (Figure 3m). Thus, naturally occurring mROS in astrocytes exert a tonic activation of

203 NRF2, which in turn contributes to maintaining GSH biosynthesis and antioxidant
204 protection of neighbor neurons.

205

206 In view that our data suggest that mROS in astrocytes, by regulating bioenergetics and
207 redox metabolism, control neuronal redox status, we next aimed to ascertain if mROS
208 reduction in astrocytes functionally affect neurons *in vivo*. Analysis of brain sections
209 obtained from the GFAP-mCAT mice by Nissl staining revealed no major anatomical
210 alterations in the brain structures (Figure 4a). However, MAP2 and TUJ1 staining
211 revealed signs compatible with dendrite alterations in neurons of the CA1 layer of the
212 hippocampus and the parietal cortex (Figure 4a) in the GFAP-mCAT but not in the
213 control mice. These data indicate that the reduction of mROS in astrocytes *in vivo*
214 negatively impacts on the neuronal structural integrity, likely affecting their function.
215 Since dendrite disruption in the hippocampus is associated with several forms of
216 cognitive decline²⁵, we subjected the GFAP-mCAT mice to a behavioral scrutiny. As
217 shown in Figure 4c, the total distance travelled in the open field test was significantly
218 decreased. Moreover, a significant increase in the ratio spent in the periphery *versus* the
219 center areas was observed along with a decrease in the number of rears (Figure 4c),
220 indicating an alteration in the explorative capacity. The motor coordination was
221 unaffected in the GFAP-mCAT mice according to the rotarod test (Figure 4d). To more
222 specifically address cognitive affection, we performed the novel object recognition test.
223 As shown in Figure 4e, the number of entries and the exploring time spent to the novel
224 object (sphere) were decreased, confirming the results obtained in the open field test.
225 Interestingly, the discrimination index was significantly reduced in the GFAP-mCAT
226 when compared with the control mice, indicating loss of short-term memory.

227

228 In summary, here we describe that astrocyte-specific down modulation of endogenous
229 mitochondrial ROS *in vivo* causes profound changes in brain energy and redox
230 metabolism eventually leading to alteration of neuronal structural integrity and
231 cognitive impairment (Figure 4f). Using this strategy, we obtained a relatively modest
232 (~25%) decrease in astrocytic mROS, suggesting that only the most sensitive targets
233 were identified. The analysis of the transcriptomics and metabolomics data identified
234 several metabolic pathways affected in the brain by mROS reduction in astrocytes,
235 which can account for the *in vivo* neuronal dysfunction. The apparently diverse
236 molecular targets and metabolic pathways affected by the decreased mROS is likely a

237 consequence of H₂O₂ unspecificity²⁶. However, we focused mainly on PPP given its
238 significant activation upon mROS down modulation, and its crucial role in controlling
239 redox balance and glucose metabolism in several paradigms^{1,9,27}. According to our data,
240 in astrocytes, physiologic mROS down modulates PPP activity and attenuates NOX-1
241 and NOX-2 mediated extracellular release of ROS. Moreover, we identify these
242 processes to be coordinated by mROS acting on at least two converging mechanisms,
243 namely HDAC4 and NRF2 transcriptional regulator signaling pathways. By keeping
244 oxidized (active) HDAC4, mROS activates miR-206 expression to repress G6PD
245 expression. On the other hand, our data also show that, through the production of
246 mROS, astrocytes keep NRF2 transcriptionally active to repress NOX-1/2 expression,
247 hence avoiding the extracellular release of ROS. This mechanism therefore represents a
248 signaling pathway connecting mitochondrial with extracellular ROS production. In
249 addition, mROS-mediated NRF2 activity in astrocytes contributes to neuronal GSH
250 replenishment, a mechanism accounting for the astrocyte-neuronal GSH shuttle that
251 keeps neuronal redox balance²². Whether these molecular mechanisms account for the
252 protection of dendrite stability and cognitive function, which we herein describe *in vivo*,
253 is a likely possibility that would require further *in vivo* studies. However, the astrocyte-
254 neuronal co-culture data showing neuronal redox stress damage by astrocyte mROS
255 attenuation strongly supports this notion. Nonetheless, we do not disregard the
256 possibility that, besides the control of redox balance, astrocytic mROS regulates
257 neuronal functions through additional mechanisms, including metabolic substrate
258 supply, likely lactate, AMP-activated protein kinase (AMPK) or ATP signaling. This
259 study is therefore first demonstrating specific physiological functions for mROS in
260 astrocytes in behaving mice.

261

262 The brain is an exceptionally vulnerable tissue to redox stress²⁸. Accordingly,
263 antioxidant compounds administration has shown protection in pre-clinical models of
264 neurological conditions closely associated with redox stress^{29,30}. Unfortunately,
265 translation of this knowledge into clinical trials have failed so far²⁹, thus challenging the
266 general notion that ROS are deleterious species. In fact, our data identifying a
267 physiological brain protective function for astrocytic high mROS helps to understanding
268 such a failure of the antioxidant therapy²⁹. Moreover, a certain level of redox stress
269 during early life in mice associates with protection against overall damage in aging as
270 well as longevity both in *C. elegans*³¹ and mice³². In sum, an increasing body of

271 evidence now suggests that ROS exert physiological functions in several tissues³³;
272 however, the potential physiological roles for mROS in the brain had not been so far
273 addressed. Our data suggest that caution should be taken when studying the
274 (patho)physiological roles of mROS in the brain, since naturally occurring high mROS
275 abundance in astrocytes coexists with low mROS in neurons¹⁰. As by analogy
276 established for exercise³⁴, cancer growth³⁵ and general mortality³⁶, administration of
277 antioxidants, at least under healthy conditions, may therefore hamper the beneficial
278 effects of astrocytic mROS for the normal brain function.

279

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292 **Authors contributions**

293 Conceived the idea: JPB

294 Performed experiments: CVG, NB, VBJ, DJB, ILF, EF, JC, GB, AA

295 Contributed materials: GB, JAE

296 Wrote the manuscript: JPB

297 Edited and approved the manuscript: All co-authors

298

299 **Declaration of Interests**

300 The authors declare no competing interests.

301

302 **Data availability statement**

303 All data generated or analysed during this study are included in this published article
304 (and its supplementary information files).

305

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393

394

395 **FIGURE LEGENDS**

396

397 **Figure 1. Generation of a conditional mouse that reduces mitochondrial ROS in**
398 **astrocytes in cultured cells and *in vivo*.**

399 (a) Schematic representation of the strategy used to generate mice expressing a
400 mitochondrial-tagged form of catalase, either constitutively (mCAT) or in astrocytes
401 (GFAP-mCAT), and their respective wild type (WT) or control littermates. C8,
402 cytochrome *c* oxidase subunit VIII-mitochondrial leading sequence; HA, human
403 influenza hemagglutinin; CAG, ubiquitous CAG promoter; STOP, transcriptional STOP
404 signal; GFAP, glial-fibrillary acidic protein promoter; CreERT2, estrogen receptor T2
405 (tamoxifen)-dependent Cre recombinase. Tamoxifen was administered intraperitoneally
406 (2 mg/25 g of body weight) for 5 consecutive days.

407 (b) Occurrence of mitochondrial-tagged catalase in astrocytes in primary culture
408 obtained from mCAT mice, as judged by western blotting.

409 (c) Mitochondrial-tagged catalase is expressed in isolated mitochondria from astrocytes
410 in primary culture obtained from mCAT mice, as judged by western blotting.

411 (d) Immunofluorescence imaging shows co-localization of mitochondrial-tagged
412 catalase with mitochondrial marker in astrocytes in primary culture obtained from
413 mCAT mice. Scale bars, 25 μ m.

414 (e) Isolated mitochondria from astrocytes in primary culture obtained from mCAT mice
415 detoxify exogenously-added H₂O₂ more efficiently than the control cells. Data are mean
416 \pm S.E.M. * $p < 0.05$ versus WT (n=3 independent experiments; Student's *t* test).

417 (f) Astrocytes in primary culture obtained from mCAT mice decreases endogenously-
418 produced H₂O₂, as determined both in isolated mitochondria and in whole cells.
419 Absolute data were 2.6 or 16.7 pmol/h x μ g protein, respectively, for the WT condition.
420 Data are mean \pm S.E.M. * $p < 0.05$ (n=3 and 4; Student's *t* test).

421 (g) Astrocytes in primary culture obtained from mCAT mice decreases endogenous
422 mitochondrial ROS whilst the mitochondrial membrane potential ($\Delta\psi_m$) remained
423 unchanged, as analyzed by flow cytometry. Data are mean \pm S.E.M. * $p < 0.05$ (n=4;
424 Student's *t* test).

425 (h) Schematic representation of the commercial immunomagnetic approach used to
426 acutely separate astrocytes (ACSA-2⁺ cells) from other neural cells (ACSA-2⁻) from the
427 adult GFAP-mCAT or control mice brain.

428 (i) *Ex vivo* analysis of mitochondrial-tagged catalase expression in astrocytes by western
429 blotting (anti-HA and anti-GFAP, left panel) and co-localization with mitochondria by
430 immunostaining (anti-HA and anti-mt-CO1, right panel; scale bars, 18 μm), as assessed
431 in the acutely isolated ACSA-2⁺ and ACSA-2⁻ cell fractions.

432 (j) *Ex vivo* analysis of H₂O₂ production in the acutely isolated ACSA-2⁺ cell fraction.
433 Absolute data were 1.7 pmol/h x μg protein in control ACSA-2⁺ cells. Data are mean \pm
434 S.E.M. *p<0.05 (n=3; Student's *t* test).

435 (k) Cells acutely dissociated from the GFAP-mCAT mouse brain, three weeks after
436 intravenous infection with AAVs expressing GFP under the control of the short GFAP
437 promoter, showed decreased endogenous mitochondrial ROS whilst the mitochondrial
438 membrane potential ($\Delta\Psi_m$) remained unchanged, as analyzed by flow cytometry. Data
439 are mean \pm S.E.M. *p<0.05 (n=4; Student's *t* test).

440 See also Supplementary Figure S1.

441

442 **Figure 2. Pentose-phosphate pathway (PPP) is increased and glycolysis decreased**
443 **in astrocytes from the mCAT mouse.**

444 (a) Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) reveals
445 increased glucose-6-phosphate dehydrogenase (*G6pd*) mRNA abundance in mCAT
446 astrocytes. Data are mean \pm S.E.M. *p<0.05 (n=3; Student's *t* test).

447 (b) Western blotting reveals increased G6PD protein abundance in mCAT astrocytes.

448 (c) The abundance of miR-206 is decreased in mCAT when compared with WT
449 astrocytes according to the RT-qPCR analysis. Data are mean \pm S.E.M. *p<0.05 (n=4;
450 Student's *t* test).

451 (d) Ectopic expression of miR-206 prevents the increase in G6PD mRNA abundance in
452 mCAT astrocytes. WT represents wild type astrocytes transfected with miR-control.
453 Data are mean \pm S.E.M. *p<0.05 *versus* WT; #p<0.05 (n=3; Student's *t* test).

454 (f) Inhibition of HDAC activity by trichostatin A (TSA, 2.5 nM) prevents the increase
455 in *G6pd* mRNA abundance in mCAT astrocytes, as revealed by RT-qPCR. Data are
456 mean \pm S.E.M. *p<0.05 *versus* WT; #p<0.05 (n=4; Student's *t* test).

457 (e) The abundance of histone deacetylase-4 (HDAC4) is higher, both in whole cells and
458 in the nuclei of mCAT astrocytes.

459 (g) Rates of ¹⁴CO₂ production from [1-¹⁴C]glucose (left) and [6-¹⁴C]glucose (middle) in
460 WT and mCAT astrocytes. The right panel shows the difference between the rates of
461 ¹⁴CO₂ production from [1-¹⁴C]- minus that from [6-¹⁴C]glucose, reflecting the rate of

462 glucose oxidation *via* the PPP. Data are mean \pm S.E.M. * $p < 0.05$ (n=3; Student's *t* test).
463 n.s., not significant.

464 (h) Lactate release is decreased in mCAT when compared with WT astrocytes. * $p < 0.05$
465 versus WT (n=3; Student's *t* test).

466 (i) Conversion of [$3\text{-}^3\text{H}$]glucose into $^3\text{H}_2\text{O}$, reflecting glycolytic rate, is decreased in the
467 mCAT when compared with the WT astrocytes. Data are mean \pm S.E.M. * $p < 0.05$ (n=4;
468 Student's *t* test).

469 See also Supplementary Figure S4.

470

471 **Figure 3. Decreased Nrf2 transcriptional activity down-modulates NADPH(H⁺)**
472 **oxidase-mediated extracellular superoxide in mCAT astrocytes and leads to**
473 **neuronal damage.**

474 (a) The NADPH(H⁺)/NADP⁺ ratio is lower in mCAT when compared with WT
475 astrocytes. Data are mean \pm S.E.M. * $p < 0.05$ (n=2; Student's *t* test).

476 (b) RT-qPCR analysis reveals increased NADPH(H⁺) oxidases 1 and 2 (NOX-1 and
477 NOX-2) mRNA abundances in mCAT when compared with WT astrocytes. Data are
478 mean \pm S.E.M. * $p < 0.05$ *versus* WT (n=3; Student's *t* test).

479 (c) Extracellular superoxide (O₂^{•-}) is higher in mCAT when compared with WT
480 astrocytes. Data are mean \pm S.E.M. * $p < 0.05$ (n=3; Student's *t* test).

481 (d) RT-qPCR analysis reveals increased Nqo1 (NAD-quinone oxidoreductase-1), Gcl
482 (glutamate-cysteine ligase, catalytic subunit), Gpx2 (glutathione peroxidase-2), Ho-1
483 (heme oxygenase-1), Trx1 (thioredoxin.1) and Nrf2, but not Prdx1 (peroxiredoxin-1)
484 mRNA abundances in mCAT when compared with WT astrocytes. Data are mean \pm
485 S.E.M. * $p < 0.05$ *versus* WT (n=3; Student's *t* test).

486 (e) Nrf2 protein abundance is lower in the nucleus of mCAT astrocytes when compared
487 with WT astrocytes.

488 (f) Ectopic expression of the constitutively active form of Nrf2, NRF2-PM rescued the
489 increased NOX-1 and -2 mRNA abundances observed in mCAT when compared with
490 WT astrocytes. Data are mean \pm S.E.M. * $p < 0.05$ *versus* WT (n=3; Student's *t* test).

491 (g) Expression of NRF2-PM rescued the increased extracellular superoxide (O₂^{•-})
492 observed in mCAT when compared with WT astrocytes. Data are mean \pm S.E.M.
493 * $p < 0.05$ *versus* WT (n=2; Student's *t* test).

494 (h) Glutamate-cysteine ligase, catalytic subunit (GCL) protein abundance is decreased
495 in the mCAT when compared with the WT astrocytes, as judged to western blotting
496 analysis.

497 (i) Total glutathione (GSH) concentration is lower in mCAT when compared with WT
498 astrocytes, without affecting oxidized glutathione. Data are mean \pm S.E.M. * $p < 0.05$
499 (n=3; Student's *t* test), absolute data were 54.46 and 43.25 nmol/mg protein for WT and
500 mCAT, respectively.

501 (j) Expression of NRF2-PM rescued the decreased GCL protein abundance observed in
502 mCAT when compared with WT astrocytes.

503 (k) Expression of NRF2-PM rescues the decreased GSH concentration observed in
504 mCAT when compared with WT astrocytes (left panel). NRF2-PM increased oxidized
505 glutathione in mCAT astrocytes (right panel). Data are mean \pm S.E.M. * $p < 0.05$ versus
506 WT; # $p < 0.05$ (n=2-3; Student's *t* test), absolute data were 54.46 nmol/mg protein for
507 WT and 39.82 and 72.21 nmol/mg protein for mCAT +/- NRF2-PM, respectively.

508 (l) Schematic representation of the co-cultures of mCAT astrocytes with WT neurons.

509 (m) The decreases in GSH concentration and oxidized glutathione, and the increases in
510 H₂O₂ production and cell death observed in WT neurons co-cultured with mCAT
511 astrocytes, are rescued by expressing NRF2-PM in mCAT astrocytes before co-
512 culturing with neurons. GSH absolute values were, 12.5 nmol/mg protein in neurons co-
513 cultured with WT astrocytes; 9.5 nmol/mg protein and 12.7 nmol/mg protein in neurons
514 co-cultured with mCAT astrocytes not (-) or transfected (+) with NRF2-PM,
515 respectively. H₂O₂ absolute values were 0.6 pmol/h x μ g protein for neurons co-cultured
516 with WT astrocytes. Data are mean \pm S.E.M. * $p < 0.05$ versus WT neurons; # $p < 0.05$
517 (n=2-3; Student's *t* test).

518 See also Supplementary Figure S4.

519

520 **Figure 4. Reduction of mitochondrial ROS in astrocytes *in vivo* alters neuronal**
521 **structural integrity and causes cognitive impairment in mice.**

522 (a) Analysis of brain sections by Nissl staining obtained from the GFAP-mCAT mice
523 revealed no major anatomical alterations in the brain structures.

524 (b) Analysis of brain sections by MAP2 and TUJ1 expression, obtained from the
525 GFAP-mCAT mice, revealed signs compatible with dendrite alterations in neurons of
526 the CA1 layer of the hippocampus and parietal cortex. Data are mean \pm S.E.M. * $p < 0.05$
527 (n=3; Student's *t* test). Scale bars, 50 μ m.

528 (c) Behavioral analysis in the open field of the GFAP-mCAT mice showed decreased
529 total distance travelled, number of rears and time spent in the central areas (B, border;
530 C, center; I, intermediate), as well as a significant increase in the ratio of the time spent
531 in the periphery *versus* the time spent in the center areas. Data are mean \pm S.E.M.
532 * $p < 0.05$ (n=13 control mice, n=9 GFAP-mCAT mice; Student's *t* test).

533 (d) Motor coordination was unaffected in the GFAP-mCAT mice according to the
534 rotarod test. Data are mean \pm S.E.M. n.s., not significant (n=7 control mice, n=9 GFAP-
535 mCAT mice; Student's *t* test).

536 (e) The number of entries and the exploring time spent to the novel object (sphere) were
537 significantly changed between the GFAP-mCAT and control mice; the discrimination
538 index was significantly reduced in the GFAP-mCAT mice when compared with the
539 control mice. Data are mean \pm S.E.M. * $p < 0.05$ (n=11 control mice, n=12 GFAP-mCAT
540 mice; Student's *t* test).

541 (f) Visual summary of the proposed overall mechanism of this study.

542

543 **Materials and Methods**

544

545 **mCAT and GFAP-mCAT mice**

546 All animal procedures we performed according to the European Union Directive
547 86/609/EEC and Recommendation 2007/526/EC, regarding the protection of animals
548 used for experimental and other scientific purposes, enforced in Spanish legislation
549 under the directive RD1201/2005. All protocols were approved by Bioethics Committee
550 of the University of Salamanca. A transgenic mouse harboring the full-length cDNA of
551 catalase fused to the cytochrome *c* oxidase subunit VIII-mitochondrial leading
552 sequence, and to the human influenza hemagglutinin (HA) for tagging purposes
553 (mitochondrial-tagged catalase) was generated by homologous recombination in the
554 *Rosa26 locus* under a C57BL/6 background upon request by genOway (Lyon, France).
555 A floxed transcriptional STOP cassette was incorporated between the mitochondrial-
556 tagged catalase cDNA and the CAG promoter (mCAT^{LoxP/+}), in order to eventually
557 obtain tissue- and time-specific expression of mitochondrial-tagged catalase *in vivo*.
558 mCAT^{LoxP/+} mice, bred under a C57BL/6-J background, was mated with CMV-Cre
559 recombinase (CMV-Cre/+ or Cre delete) mice to remove the STOP cassette to
560 constitutively express mitochondrial-tagged catalase (mCAT/+ or mCAT mice).
561 Heterozygous constitutive mCAT mice was backcrossed with C57BL/6-J mice to
562 perform *in vitro* experiments with mCAT and wild-type (+/+ or WT) littermates. To
563 express mCAT in astrocytes *in vivo*, mCAT^{LoxP/+} mice was mated with mice, under a
564 C57BL/6-J background, harboring the estrogen receptor (ERT2)-inducible (tamoxifen-
565 dependent) Cre recombinase (Cre^{ERT2}) governed by the astrocyte-specific glial fibrillary
566 acidic protein (GFAP) promoter (GFAP-Cre^{ERT2/+})¹. The progeny, namely GFAP-
567 Cre^{ERT2/+}; mCAT^{LoxP/+}, and +/+; mCAT^{LoxP/+} littermates, at the age of two months,
568 were intraperitoneally injected with 100 µl of a 20 mg/ml stock tamoxifen solution in a
569 mixture of sunflower oil and ethanol (9:1, by volume) for five consecutive days in order
570 to activate Cre recombinase activity in astrocytes *in vivo*. The resulting mice, namely
571 GFAP-Cre^{ERT2/+}; mCAT/+ (henceforth GFAP-mCAT) and +/+; mCAT^{LoxP/+} littermates
572 (henceforth Control) were used as from the age of 8 months. Animals were bred at the
573 Animal Experimentation Facility of the University of Salamanca in cages (maximum 5
574 animals/cage), and a light-dark cycle was maintained for 12 hours. Humidity was 45-
575 65% and temperature 20-25°C. Animals were fed *ad libitum* with a standard solid diet

576 (17% proteins, 3% lipids, 58.7% carbohydrates component, 4.3% cellulose, 5%
577 minerals and 12% humidity) and free access to water.

578

579 **Genotyping by polymerase-chain reaction (PCR).** The primer sequences for
580 genotyping the mCAT allele were 5'-CTCCCAAAGTCGCTCTGAGTTGTTATCA-3',
581 5'-CGATTTGTGGTGTATGTA ACTAATCTGTCTGG-3' and 5'-
582 GCAGTGAGAAGAGTACCACCATGAGTCC-3', which yielded a 778 bp band for
583 the wild type allele and a 245 bp band for the mCAT allele. To enable distinguishing the
584 non-excised recombined inducible allele containing the transcriptional STOP cassette
585 from the Cre-mediated excised allele devoid of the transcriptional STOP cassette, we
586 performed PCR using the following primers; primer 1, 5'-
587 GAGCCATCTCATCAGTAAAACTACGTCCTG-3', primer 2, 5'-
588 AGCCTCTGCTAACCATGTTCATGCC-3', and primer 3, 5'-
589 CTTACTCCACACAGGCATAGAGTGTCTGC-3', which yielded no amplification
590 product in the wild type allele, a ~729 bp band in the non-recombined mCAT allele, and
591 a ~474 bp band in the recombined mCAT allele (Supplementary Figure S1a-c). To
592 identify whether the C57BL/6 mouse strain used in this work harboured the
593 nicotinamide nucleotide transhydrogenase (*Nnt1*) mutation, we performed PCR using
594 the following primers; Nnt-COM 5'-GTAGGGCCA ACTGTTTCTGCATGA-3', Nnt-
595 WT 5'-GGGCATAGGAAGCAAATACCAAGTTG-3' and Nnt-MUT 5'-
596 GTGGAATTCCGCTGAGAGAACTCTT-3'. The amplification products were 579 bp
597 for the WT allele and 743 bp for the mutant allele² (Supplementary Figure S1e).
598 Accordingly, the behavioral phenotype of the control and GFAP-mCAT mice used in
599 this study are isogenic for *Nnt1*, hence the behavioral differences are solely due to the
600 mCAT allele.

601

602 **Primary cell cultures and co-cultures.** Astrocytes in primary culture were obtained
603 from 0-24 h old mCAT or littermate WT mouse neonates³. Cell suspension were seeded
604 in 75 cm² plastic flasks in low glucose (5.5 mM) Dulbecco's Modified Eagle's Medium
605 (DMEM) supplemented with 10% fetal bovine serum. To detach non-astrocytic cells,
606 after one week *in vitro*, the flasks were shaken at 180 r.p.m. overnight. The supernatant
607 was discarded, and the attached, astrocyte-enriched cells were pooled according to their
608 genotype and reseeded at 0.5-1 x 10⁵ cells/cm² in different size plates. At day 11 *in vitro*
609 the medium was replaced with Neurobasal A medium supplemented with 2 mM

610 glutamine, 5.5 mM glucose, 0.22 mM pyruvate and 2% of the minus antioxidant (MAO;
611 i.e., lacking vitamin E, vitamin E acetate, superoxide dismutase, catalase and
612 glutathione) B27 supplement (Life Technologies). Glucose concentrations were
613 monitored every day as from day 13 *in vitro* using a validated glucometer (Glucocard
614 G+ meter, A. Menarini diagnostics) and adjusted to 5.5 mM from a 1 M glucose stock
615 solution. Astrocytes were used for experiments on day 15 *in vitro*. Primary cultures of
616 mice cortical neurons were prepared from E15.5 days WT mouse, seeded at 2.0×10^5
617 cells/cm² in different size plastic plates coated with poly-D-lysine (10 µg/ml) and
618 incubated in Neurobasal A supplemented with 2 mM glutamine, 5.5 mM glucose, 0.22
619 mM pyruvate and 2% B27 MAO supplement. Cells were incubated at 37 °C in a
620 humidified 5% CO₂-containing atmosphere. At 72 hours after plating, medium was
621 renewed and cells used at day 7. To obtain astrocyte-neuronal co-cultures, astrocytes at
622 8 days *in vitro* were re-seeded on semipermeable polyester Transwell® membrane
623 inserts (0.4 µm pore size, Corning) and, at day 11 *in vitro*, astrocyte-containing inserts
624 were placed over 3 days *in vitro* neurons and co-cultured in Neurobasal A supplemented
625 with 2 mM glutamine, 5.5 mM glucose, 0.22 mM pyruvate and 2% B27 MAO
626 supplement for 4 more days.

627

628 **Cell transfections.** Cells were transfected using Lipofectamine LTX-PLUS Reagent
629 (Life Technologies) according with manufacturer's protocol. Plasmid was a
630 T395D/S433D/T439D triple phosphomimetic, constitutively active GFP-NRF2 cDNA
631 fusion construct³. Cells were used after 72 hours of transfection. To overexpress miR-
632 206, 3 nM of Ambion Pre-miRTM miRNA precursor for miR-206 (PM10409, Cat.
633 #AM17100) or the Ambion Pre-miRTM negative control (Cat #AM17110) was delivered
634 into the cells using Lipofectamine RNAiMAX (Life Technologies). Cells were used
635 after 24 hours of transfection.

636

637 **Mitochondria isolation.** Mitochondria were obtained according to a previously
638 published protocol⁴. Briefly, cells (12-100 millions) were collected, cell pellets frozen at
639 -80 °C and homogenized (10 strokes) in a glass-teflon Potter-Elvehjem homogenizer in
640 Buffer A (sucrose 83 mM; MOPS 10 mM; pH 7.2). The same volume of Buffer B
641 (sucrose 250 mM; MOPS 30 mM) was added to the sample, and the homogenate was
642 centrifuged (1,000 x g, 5 min) to remove unbroken cells and nuclei. Centrifugation of
643 the supernatant was then performed (12000 x g, 2 min) to obtain the mitochondrial

644 fraction, which was washed in Buffer C (sucrose 320 mM; EDTA 1 mM; Tris-HCl 10
645 mM; pH 7.4). Mitochondria were suspended in Buffer D (6-aminohexanoic acid 1M;
646 Bis-Tris-HCl 50 mM; pH 7.0).

647

648 **Mitochondrial catalase activity determination.** This was measured in isolated
649 mitochondria according to a previously published protocol⁵ based on the ability of
650 catalase to eliminate exogenous H₂O₂. Mitochondrial samples (5 µg of protein) were
651 incubated, in a final volume of 50 µl of 0.1 M phosphate buffer, pH 7.4, with 25, 50 and
652 100 µM H₂O₂ for 0, 5, 10, 15 and 20 minutes. Reactions were stopped by adding HCl
653 (37% final concentration, vol/vol). Afterwards, 3.2 mM (NH₄)₂Fe(SO₄)₂ and 180 mM
654 KSCN were added and the samples incubated for 10 min at room temperature in the
655 dark. The absorbance at 492 nm was measured using a Varioskan® Flash (Thermo
656 Fisher) and the concentration of the residual H₂O₂ was calculated using a standard
657 curve.

658

659 **Mitochondrial ROS.** Mitochondrial ROS was determined using the fluorescent probe
660 MitoSox™ (Life Technologies). Cells, astrocytes in primary culture or adult brain cell
661 suspensions, were incubated, respectively, with 2 or 3 µM of MitoSox™ for 30 min at
662 37°C in a 5% CO₂ atmosphere in HBSS buffer (NaCl 134.2 mM; KCl 5.26 mM;
663 KH₂PO₄ 0.43 mM; NaHCO₃ 4.09 mM; Na₂HPO₄·2H₂O 0.33 mM; glucose 5.44 mM;
664 HEPES 20 mM; CaCl₂·2H₂O 4 mM; pH 7.4). Cells were then washed with PBS
665 (phosphate-buffered saline, 0.1 M) and, in the case of cultured cells, collected by
666 trypsinization. MitoSox™ fluorescence intensity was assessed by flow cytometry
667 (FACScalibur flow cytometer, BD Biosciences) and expressed in arbitrary units.

668

669 **H₂O₂ determination.** For H₂O₂ assessments, Amplex™ Red Hydrogen
670 Peroxide/Peroxidase Assay Kit (Life Technologies) was used. Cultured astrocytes or
671 ACSA-2⁺ cells were reseeded onto 96 well plates at day 8 in culture. On day 15,
672 attached astrocytes were washed with PBS and incubated in KRPG buffer (NaCl 145
673 mM; Na₂HPO₄ 5.7 mM; KCl 4.86 mM; CaCl₂ 0.54 mM; MgSO₄ 1.22 mM; glucose 5.5
674 mM; pH 7.35) in the presence of 9.45 µM Amplex™ Red containing 0.1 U/ml of
675 horseradish peroxidase. Luminescence was recorded for 2 hours at 30 min intervals
676 using a Varioskan® Flash (Thermo Scientific) (λ_{exc} = 538 nm; λ_{em} = 604 nm). Slopes

677 were used for the calculations of the rates of H₂O₂ formation, and the results were
678 expressed as fold change.

679

680 **Extracellular superoxide (O₂^{•-}) determination.** The production of extracellular O₂^{•-}
681 was measured following a previous published protocol⁶. Briefly, astrocytes seeded in
682 96-well plates were washed twice with KRPG and incubated in the presence of 120 μM
683 of oxidized cytochrome c (Sigma-Aldrich) for 4 hours at 37°C. Absorbance at 550 nm
684 was recorded and O₂^{•-} concentration calculated using the extinction coefficient of the
685 reduced form of cytochrome *c* and normalized to protein concentration. Results were
686 expressed as fold change, arbitrarily assigning the value of 1 to control cells.

687

688 **Determination of total glutathione concentrations and glutathione oxidized status.**

689 Cells were lysed with 1% (w/v) of sulfosalicylic acid, centrifuged at 13,000 x *g* for
690 5 min at 4°C, and the supernatants were used for the determination of total glutathione
691 (i.e., reduced glutathione concentration plus twice the concentration of oxidized
692 glutathione), using oxidized glutathione (GSSG; 0-50 μM) as standard, as described
693 previously⁷. Total glutathione was measured in reaction buffer (0.1 mM NaHPO₄, 1
694 mM EDTA, 0.3 mM DTNB, 0.4 mM NADPH, glutathione reductase 1 U/ml, pH 7.5)
695 by recording the increase in the absorbance at 405 nm after the reaction of reduced
696 glutathione with 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) for 2.5 min at 15 sec
697 intervals using a Varioskan® Flash (Thermo Fisher). GSSG was quantified after
698 derivatization of GSH with 2-vinylpyridine using similarly treated GSSG standards (0–
699 5 μM), and results were expressed as the oxidized glutathione status (GSSG/GSH x
700 100).

701

702 **Active caspase-3 determination.** This was used to estimate apoptosis. A fluorimetric
703 caspase 3 assay kit (Sigma-Aldrich) was used following the manufacturer's protocol.
704 This assay is based on the hydrolysis of the peptide substrate Ac-DEVD-AMC (acetyl-
705 Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) by caspase-3, which results in the
706 release of fluorescent 7-amino-4-methylcoumarin (AMC). In brief, cells were lysed
707 with 50 mM HEPES, 5 mM CHAPS, 5 mM DTT, pH 7.4 for 20 min on ice, and the
708 assay buffer containing the Ac-DEVD-AMC substrate (20 mM HEPES, 2 mM EDTA,
709 0.1% CHAPS, 5 mM DTT, 16 μM Ac-DEVD-AMC, pH 7.4) was added. Aliquots of
710 200 μl were transferred to a 96-wells plate and the fluorescence recorded for 2 hours at

711 20 min intervals at 37 °C ($\lambda_{exc}=360$ nm, $\lambda_{em}=460$ nm). CSP-3 activity was determined as
712 AMC release rate extrapolating the slopes to those obtained from an AMC standard
713 curve. Results were expressed as fold change, arbitrarily assigning the value of 1 to
714 control cells.

715

716 **Determination of PPP and glycolytic fluxes.** These were measured in 8 cm² flasks of
717 adherent cells at 60-70% confluence containing a central microcentrifuge tube with
718 either 0.8 ml benzethonium hydroxide (Sigma) for ¹⁴CO₂ equilibration or 1 ml H₂O for
719 ³H₂O equilibration. Incubations were carried out in KRPG containing 5 mM D-glucose
720 at 37 °C in the air-thermostated chamber of an orbital shaker (Forma Benchtop Orbital
721 Shaker, Model 420, Thermo Fischer). In order to ensure adequate oxygen supply for
722 oxidative metabolism throughout the incubation period, flasks were filled with oxygen
723 before being sealed. To measure the carbon flux from glucose through the PPP, cells
724 were incubated in KRPG (5 mM D-glucose) buffer supplemented with 0.5 μCi D-[1-
725 ¹⁴C]glucose or [6-¹⁴C]glucose for 90 min as previously described^{8,9}. Incubations were
726 then terminated by the addition of 0.2 ml 20% perchloric acid (Merck Millipore) for 40
727 min before the benzethonium hydroxide (containing ¹⁴CO₂) was removed and the
728 radioactivity measured with a liquid Scintillation Analyzer (Tri-Carb 4810 TR,
729 PerkinElmer). PPP flux was calculated as the difference between ¹⁴CO₂ production from
730 [1-¹⁴C]glucose (which decarboxylates through the 6-phosphogluconate dehydrogenase-
731 catalyzed reaction) and that of [6-¹⁴C]glucose (which decarboxylates through the
732 tricarboxylic acid cycle)^{8,10}. Glycolytic flux was measured by assaying the rate of ³H₂O
733 production from [3-³H]glucose using a similar method but instead incubating cells with
734 5 μCi D-[3-³H]glucose in KRPG buffer per flask for 120 minutes as previously
735 described^{8,9}. Incubations were then terminated with 0.2 ml 20% perchloric acid, and
736 cells further incubated for 96 hours with a microcentrifuge tube, containing H₂O,
737 suspended above the cells to allow ³H₂O equilibration. The ³H₂O was then measured by
738 liquid scintillation counting (Tri-Carb 4810 TR, PerkinElmer). Under these
739 experimental conditions, 75% of the produced ¹⁴CO₂ or 28% of the produced ³H₂O were
740 recovered, which were used for the calculations as previously established⁹.

741

742 **Lactate determination.** Lactate concentrations were measured in the culture medium
743 spectrophotometrically⁸ by determining the increments in absorbance of the samples at

744 340 nm in a mixture containing 1 mM NAD⁺ and 22.5 U/ml lactate dehydrogenase in
745 0.25 M glycine/0.5 M hydrazine/1 mM EDTA buffer at pH 9.5.

746

747 **NADPH/NADP⁺ ratio determination.** This was performed using a colorimetric
748 NADP⁺/NADPH assay kit (Abcam). Cells were re-suspended in 500 µl of
749 NADP⁺/NADPH extraction buffer, vortexed and centrifuged at 14,000 x g for 5 min to
750 remove insoluble material. The supernatant was used for NADPH plus NADP⁺
751 measurement. NADPH was determined in 200 µl of the supernatant after being heated
752 at 60 °C for 30 min in order to decompose NADP⁺. The actual NADP⁺ and NADPH⁺
753 concentrations were calculated by extrapolating the values to a NADPH standard curve
754 (0-100 pmol/well).

755

756 **Mitochondrial membrane potential.** The mitochondrial membrane potential ($\Delta\Psi_m$)
757 was assessed using MitoProbe™ DiIC₁(5) (Life Technologies) (50 nM) by flow
758 cytometry (FACScalibur flow cytometer, BD Biosciences). For this purpose, suspended
759 cells were incubated with the probe at 37 °C for 30 min in the presence of PBS. $\Delta\Psi_m$
760 values were expressed in relative percentages from arbitrary units after subtracting the
761 potential value obtained in the presence of CCCP (10 µM, 15 min) in each sample.

762

763 **Intravenous injections of adeno-associated viral vectors (AAVs).** We used an AAVs
764 infection approach to distinguish adult astrocytes *in vivo*. To do this, we used the AAV-
765 PHP.eB capsid¹¹ that efficiently transduce the central nervous system following
766 intravenous injection. The AAV express a green fluorescent protein (GFP) driven by the
767 short astrocyte-specific GFAP promoter (gfa-ABC₁D-GFP)¹². Adult mice (14 months
768 old) were briefly anesthetized with sevoflurane (Sevorane. AbbVie, Spain) at 6% for
769 initiation followed by ~3% for maintenance in air with supplement O₂ and NO₂ (0.4 and
770 0.8 litres/min, respectively) using a gas distribution column (Hersill H-3, Spain) and a
771 vaporizer (InterMed Penlons Sigma Delta, UK). A 50 µl aliquot of a PBS solution
772 containing 0.001% Pluronic® F-68 (Sigma-Aldrich) and 5 x 10¹⁰ VG (viral genomes) of
773 AAV-PHP.eB-gfa-ABC₁D-GFP was administered in the retro-orbital venous sinus. We
774 chose this intravenous route because of the higher success rate observed when
775 compared with the tail or temporal ones¹³. The total duration of the procedure per
776 mouse was less than 10 min, including anesthesia and recovery. Three weeks after
777 infection, brains were extracted and a single cell suspension was achieved by

778 trypsinization and smooth mechanical resuspension¹⁴. The cell suspension was
779 incubated with MitoSoxTM and MitoProbeTM DiIC₁(5), as described above, to assess
780 mitochondrial ROS and mitochondrial membrane potential, respectively, in GFP⁺ cells
781 by flow cytometry (FACScalibur flow cytometer, BD Biosciences).

782

783 **Immunomagnetic purification of neurons and astrocytes from adult brain.** Mouse
784 adult brain was dissociated using the adult mouse brain dissociation kit (Miltenyi
785 Biotec). Dissociated cells after removal of debris and red blood cells were separated
786 using either the astrocyte-specific anti-ACSA-2 Microbead Kit or the neuron-specific
787 Neuron Isolation Kit, according to manufacturer's protocol (MACS® technology). We
788 confirmed the identity of the isolated fractions by Western blotting against neuronal
789 (MAP2) or astrocytic (GFAP)-specific markers¹⁴.

790

791 **Total protein extraction.** To obtain total cell protein extracts, cells were washed with
792 cold PBS and lysed in RIPA buffer (1% sodium dodecylsulphate, 10 mM
793 ethylenediaminetetraacetic acid (EDTA), 1 % v/v Triton Tx-100, 150 mM NaCl, 10
794 mM Na₂HPO₄, pH 7.0), supplemented with phosphatase (1 mM Na₃VO₄, 50 mM NaF)
795 and protease (100 µM phenylmethylsulfonyl fluoride (PMSF), 50 µg/ml aprotinin, 50
796 µg/ml leupeptin, 50 µg/ml pepstatin, 50 µg/ml anti-papain, 50 µg/ml amastatin, 50
797 µg/ml bestatin and 50 µg/ml soybean trypsin inhibitor) inhibitor cocktail, and boiled for
798 5 min. Extracts were then centrifuged at 13.000 x g for 10 min and the supernatant
799 collected for analysis.

800

801 **Cytosolic and nuclear protein fractionation.** To obtain cytosolic and nuclear fractions
802 from cell cultures, a previously reported protocol³ was used, with some modifications.
803 This method first uses a mildly aggressive solution (cytosolic buffer; 10 mM HEPES,
804 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, NP-40 0.1%, vol/vol, 1.5 M sucrose and
805 protease and phosphatase inhibitor cocktail, pH 7.9) that fragmentizes cellular
806 membranes maintaining the integrity of the nuclei, which are lysed using a more
807 aggressive solution (nuclear buffer; 50 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl mM,
808 0.5 mM NaCl, 1 mM EDTA, NP-40 1%, v/v and protease and phosphatase inhibitor
809 cocktail, pH 7.9) to obtain nuclear proteins exclusively.

810

811 **Cytosolic and mitochondrial protein fractionation.** To obtain cytosolic and
812 mitochondrial fractions from cultured cells, a differential centrifugation protocol was
813 used¹⁵. Cells grown in 145 cm² plates were collected in Mitochondria Isolation Buffer
814 (MIB, 320 mM sucrose, 1 mM potassium EDTA, 10 mM Tris-HCl and the protease and
815 phosphatase inhibitor cocktail, pH 7.4). Cells were centrifuged at 600 x g for 5 min and
816 the pellet re-suspended in MIB. After 18 stroke homogenization steps in a Teflon pestle
817 attached to a Potter-Elvehjem homogenizer, the homogenate was centrifuged three
818 times at 1.300 x g for 10 min, keeping the supernatants in every step. The mitochondrial
819 pellet resulted from the final centrifugation (at 17.000 x g for 12 min) was re-suspended
820 in RIPA buffer, and the supernatant, containing the cytosolic fraction, was kept on ice.

821

822 **Primary antibodies for Western blotting and immunocytochemistry.**

823 Immunoblotting was performed with anti-GFAP (1/1000) (G9269, Sigma), anti-catalase
824 (1/1000) (PA5-23246, Thermo Scientific), anti-GAPDH (1/40000) (4300, Ambion)
825 anti-G6PD (1/1000) (A9521, Sigma), anti-GCL (1/1000) (sc-22755, Santa Cruz
826 Biotechnologies), anti-HA tag (1/10000) (26183, Thermo Scientific), anti-HA-tag
827 (1/1000) (C29F4, Cell Signaling), anti-HDAC4 (1/1000) (2072, Cell Signaling), anti-
828 Lamin B (1/500) (sc-374015, Santa Cruz Biotechnologies), anti-NRF2 (1/1000)
829 (ab62352, Abcam), anti-VDAC (1/1000) (PC548, Calbiochem), anti-MT-CO1 (1/1000)
830 (ab14705, Abcam) and anti- β -ACTIN (1/30000) (A5441, Sigma).

831

832 **Western blotting.** Cells were lysed in RIPA buffer (1% SDS; 2 mM EDTA; 12.5 mM
833 Na₂HPO₄; 1% triton X-100; 150 mM NaCl; pH 7), supplemented with phosphatase (1
834 mM Na₃VO₄, 50 mM NaF) and protease (100 μ M phenylmethylsulfonyl fluoride
835 (PMSF), 50 μ g/ml aprotinine, 50 μ g/ml leupeptine, 50 μ g/ml pepstatin, 50 μ g/ml anti-
836 papain, 50 μ g/ml amastatin, 50 μ g/ml bestatin and 50 μ g/ml soybean trypsin inhibitor)
837 inhibitor cocktail, 100 μ M phenylmethylsulfonyl fluoride and phosphatase inhibitors (1
838 mM o-vanadate). Samples were boiled for 5 min. Aliquots of cell lysates (50 μ g protein,
839 unless otherwise stated) were subjected to sodium dodecyl sulfate-polyacrylamide
840 (SDS-PAGE) electrophoresis on an 8 or 10% acrylamide gel (MiniProtean, Bio-Rad)
841 including PageRuler Plus Prestained Protein Ladder (Thermo). The resolved proteins
842 were transferred electrophoretically into nitrocellulose membranes (Amersham protran
843 premium 0.45 nitrocellulose, Amersham). Membranes were blocked with 5% (wt/vol)
844 low-fat milk in 20 mM Tris, 150 mM NaCl, and 0.1% (w/v) Tween 20, pH 7.5, for 1 h.

845 Subsequently, membranes were immunoblotted with primary antibodies overnight at
846 4°C. After incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG
847 (Santa Cruz Biotechnologies), goat anti-mouse IgG (Sigma and BioRad), mouse anti-
848 rabbit IgG (Sigma), or rabbit anti-goat IgG (Abcam) (all at 1/10000 dilution, except
849 goat anti-rabbit and mouse anti-rabbit, which were at 1/4000 and 1/5000, respectively),
850 membranes were immediately incubated with the enhanced chemiluminescence kit
851 WesternBright ECL (Advansta) or SuperSignal™ West Femto Maximum Sensitivity
852 Substrate (Thermo Scientific™), before exposure to Fuji Medical X-Ray film
853 (Fujifilm), and the autoradiograms scanned. Three to four biologically independent
854 replicates were always performed, though only one representative western blot is shown
855 in the main article. The protein abundances of all western blots per condition were
856 measured by densitometry of the bands on the films using ImageJ 1.48u4 software
857 (National Institutes of Health, USA), were normalized, and the resulting values were
858 used for the statistical analysis³.

859

860 **Real-time quantitative polymerase chain reaction (RT-qPCR).** This was performed
861 in total RNA samples, purified from astrocytes using the GenElute Mammalian Total
862 RNA Miniprep Kit (Sigma), following the manufacturer's protocol. Amplifications
863 were performed in 100 ng of RNA, using Power SYBR Green RNA-to-CT 1-Step kit
864 (Applied Biosystems). Forward and reverse primers were, respectively (Thermo
865 Scientific, Offenbach, Germany), 5'- AGAGTCATGAGCTGCCTGAC-3' and 5'-
866 AGAGTCATGAGCTGCCTGAC-3' (*β-Actin*), 5'-ATGCAGGCCAACCGTCTATT-3'
867 and 5'-TGTCTGACTCATGCAGGTCT-3' (*G6pd*), 5'-
868 GGCACAAGGACGTGCTCAAGT-3' and 5'- TTTGTCCTCTCCCCCTTCTC-3'
869 (*Gcl*), 5'- CGGGACTACAACCAGCTCAAT-3' and 5'-
870 TCCGAAGTGGTTGCAAGGGAA-3' (*Gpx2*), 5'-AGCACAGGGTGACAGAAGAG-
871 3' and 5'- GAGGGACTCTGGTCTTTGTG-3' (*Ho-1*), 5'-
872 AAATTCCAGCGTGCCGACAA-3' and 5'-AACCAGGCAAAGGCACCTGT-3'
873 (*Nox1*), 5'-ATGCAGGAAAGGAACAATGC-3' and 5'-
874 GTGCACAGCAAAGTGATTGG-3' (*Nox2*), 5'-GCAGGACATGGATTTGATTGA-3'
875 and 5'- GTCAAACACTTCTCGACTTAC-3' (*Nrf2*), 5'-
876 GGGGACATGAACGTCATTCTCT-3' and 5'- AAGACCTGGAAGCCACAGAAGC-
877 3' (*Nqo1*), 5'-CAAGTGA TTGGCGCTTCTGT-3' and 5'-
878 TGTTCATGGGTCCCAATCCT-3' (*Prdx1*) and 5'-GGTGTGGACCTTGCAAATG-

879 3' and 5'- GGCAGTCATCCACATCCACTT-3' (*Trx1*). The mRNA abundance of each
880 transcript was normalized to the β -actin mRNA abundance obtained in the same sample.
881 The resulting normalized values in astrocytes were expressed as the fold change *versus*
882 the corresponding normalized values in WT astrocytes.

883

884 **Immunofluorescence.** Astrocytes in culture were grown on glass coverslips, and
885 freshly isolated adult astrocytes (Miltenyi protocol) were seeded in Lab-Tek® Chamber
886 Slide™ system previously treated with poly-D-Lysine and left attaching during 4 days
887 previous the immunocytochemistry protocol. Attached cells were fixed with 4%
888 paraformaldehyde (vol/vol) in PBS for 20 min and washed with PBS. Subsequently,
889 astrocytes were incubated in 5% goat serum, 1% BSA PBS-Tx 0.2% for 1 hour at room
890 temperature. Afterwards, they were incubated with the primary antibody in 2% goat
891 serum, 1% BSA, PBS-Tx 0.2% overnight at 4°C. On the following day, astrocytes were
892 washed with PBS-Tx 0.2% and incubated with the secondary antibody and the nuclear
893 marker DAPI (Sigma, D9542, 1/10,000) in 2% goat serum, 1% BSA, PBS-Tx 0.2% for
894 1 hour at room temperature. Subsequently, glass coverslips were placed on a glass slide
895 using SlowFade® (Molecular Probes) in order to avoid fluorescence loss. Primary
896 antibodies used were anti-Heat shock protein 60 (HSP60) (1/500) (ab46798, Abcam),
897 anti-HA tag (1/1000) (2367, Cell Signaling) and anti-NRF2 (1/500) (ab62352, Abcam),
898 anti-HA-tag (1/1000) (C29F4, Cell Signaling), anti-MT-CO1 (1/1000) (ab14705,
899 Abcam). Secondary antibodies used were Cy2 goat anti-mouse (1/500) (115-225-003),
900 Cy2 goat anti-rabbit (1/500) (111-225-144), Cy3 goat anti-mouse (1/500) (115-165-
901 003) and Cy3 goat anti-rabbit (1/500) (111-165-003) (all from Jackson
902 ImmunoResearch). Confocal microscopy images were obtained using a spinning disk
903 confocal microscopy Olympus IX81 and processed with Adobe Photoshop cs5.5
904 software and ImageJ.

905

906 **miRNA expression analysis.** To obtain a micro-RNA (miRNA) enriched fraction, the
907 miRNeasy kit (Qiagen) was used following the manufacturer's protocol. To perform the
908 miRNA reverse transcription, 50 ng of miRNA-enriched fraction was mixed with 0.1 μ l
909 of 100 nM dNTP, 0.14 μ l of RNase inhibitor (20 U/ μ l), 0.67 μ l of reverse transcriptase
910 (50 U/ μ l), 1 μ l RT-Buffer 1X and 4.5 μ l of primer pool in a final volume of 10 μ l. All
911 reagents were purchased from Applied Biosystems. Samples were incubated for 5 min

912 on ice, 30 min at 16°C, 30 min at 42°C and finally 5 min at 85°C. Pre-PCR amplification
913 reaction was done at 5 µl containing 2.5 µl TaqMan PreAmp Master Mix (2X), 1.25 µl
914 of pooled TaqMan assay mix (0.2X each assay) and 1.25 µl of cDNA. The pre-
915 amplification PCR was performed at one cycle 95°C for 10 min, 10 cycles at 95°C for
916 15 sec followed by 4 min at 60°C. All reagents were purchased from Applied
917 Biosystems. Relative miRNA expression was evaluated using a 48.48 dynamic array
918 (Fluidigm Corporation). miRNA expression analysis was carried out using the 48.48
919 dynamic array (Fluidigm Corporation) following the manufacturer's protocol. Different
920 miRNA expression was calculated using the comparative method of C_t ($\Delta\Delta C_t$), as
921 previously described and analysed at the ADN Sequencing Service of Nucleus,
922 University of Salamanca. TaqMan assays used were mmu-miR-202-5p (endogenous
923 control) and hsa-miR-206 (Thermo Scientific).

924

925 **Transcriptomic microarray.** Neurons and astrocytes were acutely dissociated, using
926 the immunomagnetic approaches described as above, from 14 months-old control or
927 GFAP-mCAT mice (n=4 for each condition). Total RNA samples were extracted and
928 purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's
929 instructions. RNA integrity and quantity were assessed with an Agilent 2100
930 Bioanalyzer. Whole-genome expression analysis was carried out using Affymetrix
931 Clariom™ D Array for mouse (Thermo Fisher Scientific). The data were processed by
932 Affymetrix Transcriptome Analysis Console (TAC) Software with default setting of
933 Gene + Exon-SST-RMA summarization method to calculate the expression level for
934 each probeset of the array. The difference in expression was calculated using the median
935 value in log base 2 from each probeset over all the previously normalized chips. TAC
936 was also used to generate heatmaps and volcano plots. A list of probesets/transcripts
937 with a statistically significant differential expression was then generated with the
938 criteria that the fold change was higher than +/- 1.3 and the p-value 0.05 (Limma,
939 eBayes analysis). The generated transcripts sublist were functionally annotated using
940 the online bioinformatics resources Database for Annotation, Visualization and
941 Integrated Discovery (DAVID version 6.8) and the Integrated Molecular Pathway Level
942 Analysis (IMPALA version 11), with default settings. The enriched functional-related
943 gene groups were filtered according to their relevance and the selected clusters were
944 referred with the corresponding terms (for example, "metabolism", "disulfide bond")

945 extracted from the annotated file and derived from different Database resources
946 including Gene Ontology terms, bio-pathways, protein functional domains and others.

947

948 **Metabolomic analysis.** One hemisphere of 14 months-old control or GFAP-mCAT
949 mice (n=7 for each condition) was snap frozen in liquid nitrogen and used for
950 untargeted metabolomic analysis (Metabolon, Morrisville, NC, USA) according to the
951 proprietary methodology to provide a biochemical profiling and statistical analysis
952 associated with compared conditions. The Ultrahigh Performance Liquid
953 Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) analysis detected 540
954 metabolites. Raw data were extracted, peak-identified, QC processed, curated and
955 normalized by Metabolon service. Following log transformation and imputation of
956 missing values, with the minimum observed value for each compound, Welch's two-
957 sample *t*-test were used to identify compounds significantly different between
958 experimental groups. Using these filters, we accepted a high estimate of the false
959 discovery rate ($q\text{-value} \leq 0.50$), although other considerations were implemented to
960 determine whether a result merited further scrutiny. Such evidence included (i)
961 significance for another dimension of the study, (ii) inclusion in a common pathway
962 with a highly significant compound, (iii) residing in a similar functional biochemical
963 family with other significant compounds or (iv) correlation with other experimental
964 approaches. Cytoscape 3.6.0 Software was used for visualization and analysis of
965 biological networks. Graphs corresponding to statistical analysis were carried out with
966 the online tool MetaboAnalyst 4.0, and integration with transcriptomics microarray data
967 was performed with the Integrated Molecular Pathway Level Analysis (IMPALA
968 version 11), with default settings. Only pathways with further evidence were
969 highlighted.

970

971 **Mice perfusion and immunohistochemistry.** Male mice (14 months-old) were
972 anesthetized by intraperitoneal injection of a mixture of xylazine hydrochloride
973 (Rompun; Bayer) and ketamine hydrochloride/chlorbutol (Imalgene; Merial) (1:4) at 1
974 ml per kg of body weight, and then perfused intra-aortically with 0.9% NaCl followed
975 by 5 ml/g body weight of Somogyi (Paraformaldehyde, 4% (w/v) and picric acid, 0.2%
976 (vol/vol), in 0.1M PBS at pH 7.4. After perfusion, brains were dissected out sagittally in
977 two parts and post-fixed using Somogyi during 2 hours at room temperature. Brain
978 blocks were rinsed successively for 10 min, 30 min, and 2 hours with 0.1M PBS

979 solution and cryoprotected in 10%, 20% and 30% (w/v) sucrose in PBS sequentially,
980 until they sank. After cryoprotection, 40- μ m-thick sagittal sections were obtained with a
981 freezing-sliding cryostat (Leica; CM1950 AgProtect). Sectioning of WT and mCAT
982 brains were performed under the same conditions and sessions. The sections were
983 collected serially in a 12 well plate in 0.1 M PBS and rinsed 3 times for 10 min in 0.1M
984 PBS and used for subsequent immunohistochemistry and Nissl staining. The section-
985 containing wells that were not used were kept in freezer mix (polyethylene glycol, 30%
986 by vol and glycerol 30% by vol in 0.1 M PBS) at -20°C. For immunohistochemistry,
987 sections were incubated sequentially in (i) 5 mg/ml sodium borohydride in PBS for 30
988 min (to remove aldehyde autofluorescence); (ii) three PBS washes of 10 min each; (iii)
989 1:500 anti-MAP2 (AP-20; Abcam #11268) or 1:500 anti-beta III Tubulin (Abcam
990 #18207) in 0.02% Triton X-100 (Sigma-Aldrich) and 5% goat serum (Jackson
991 Immuno-Research) in 0.1 M PBS for 72 hours at 4 °C; (iv) three PBS washes of 10 min
992 each; (v) fluorophore conjugated secondary antibodies, 1:500 Cy2 goat anti-mouse or
993 1:500 Cy3 goat anti-rabbit (Jackson Immuno-Research) in PBS for 2 hours at room
994 temperature; and (vi) 0.5 μ g/ml DAPI in PBS for 10 min at room temperature. After
995 rinsing with PBS, sections were mounted with Fluoromount (Sigma-Aldrich) aqueous
996 mounting medium and lamelles couvre-objects (MenzelTM, Thermo)¹⁶.

997

998 **Nissl staining.** Sections obtained as previously described were mounted in slides and
999 left to dry overnight at room temperature. Slides were immersed in a Coplin jar in
1000 absolute ethanol and chloroform mix (1:1) overnight, and sequentially immersed for 5
1001 min each in a battery of ethanol solutions at decreasing concentrations, starting from
1002 xylol down to distilled water for rehydration. Then, the slides were immersed again in
1003 Nissl dye, consisting of 0.1% (wt/vol) cresyl violet and 0.3% (vol/vol) glacial acetic in
1004 water, for 10 min at room temperature. Afterwards, sides were washed under tap water,
1005 distilled water and, finally, of ethanol solutions at creasing concentrations, starting from
1006 96% ethanol down to xylol for deshydration, and mounted in Entellan[®] solution
1007 (Sigma).

1008

1009 **Imaging and quantification.** Sections were examined with epifluorescence and
1010 appropriate filter sets using an inverted microscope (Nikon; Eclipse[®] Ti-E) equipped
1011 with a pre-centered fiber illuminator (Nikon; Intensilight C-HGFI), B/W CCD digital
1012 camera (Hamamatsu; ORCA-E.R.) and Plan 40 \times /0.65 ∞ /0.17 WD 0.56 objective.

1013 Confocal images were acquired using a spectral laser confocal microscope (TCS-SL;
1014 Leica Microsystems). Large fields of view were acquired with an HCX 40×/0.75 PH2
1015 Plan Fluotar with a pixel size of ~300 nm at a scan speed of 400 Hz using three-line
1016 averages. High-resolution images were acquired using an HCX 63×/1.4 – 0.60 Plan Apo
1017 CS oil objective with a pixel size of 100 nm and a z-step size of 290 nm at a scan speed
1018 of 400 Hz using three lines averages. Immunohistochemical digital images were used to
1019 analyze different proteins staining in the three most sagittal sections per animal from
1020 three different animals per condition (n = 3). Images were exported into ImageJ in tiff
1021 format for processing. Before image analysis, a maximum-intensity projection over z-
1022 series projections spanning 15 to 19 μm was performed. Images were converted to gray
1023 scale 8-bit images and brightness/contrast was adjusted using the ImageJ “auto”
1024 function. Staining was automatically delineated using the “auto setting threshold”
1025 (default method) and “dark background” functions of ImageJ. Threshold images were
1026 subsequently quantified as percent area (area fraction) using the “analyze-measure”
1027 function, which represents the percentage of pixels in the image that have been
1028 highlighted (% area)¹⁶.

1029

1030 **Behavioral tests.** Male mice (8-10 months old) were left to acclimatize in the room for
1031 not less than 15 min at the same time slot of the day (2 pm-8 pm). Tracking was carried
1032 out once at a time and carefully cleaning the apparatus with 70% ethanol between trials
1033 to remove any odor cues. An ANY-box® core was used, which contained a light grey
1034 base and an adjustable perpendicular stick holding a camera and an infrared photo-beam
1035 array to track the animal movement and to detect rearing behavior, respectively. Mouse
1036 movements were tracked with the ANY-maze® software and the AMi-maze® interface
1037 to register all parameters described subsequently. For the Open Field test, a 40 cm x 40
1038 cm x 35 cm (w, d, h) black infrared transparent Perspex insert was used, and the arena
1039 was divided in three zones, namely border (8 cm wide), center (16 % of total arena) and
1040 intermediate (the remaining area). The test lasted for 10 min, the distance travelled, the
1041 number of rearings and the time spent in each zone were measured. Rotarod test
1042 (Rotarod apparatus, Model 47600, Ugo Basile) was used to analyze motor balance and
1043 coordination. Male mice were previously trained during three consecutive days, two
1044 days before the test. The rotarod conditions were a gradually acceleration from 4 to 25
1045 r.p.m., reaching the final speed at 270 sec. To analyze the short-time memory, we used
1046 the Novel Object Recognition test (Stoelting) in a 40 cm x 40 cm x 35 cm (w, d, h) core

1047 with black infrared transparent Perspex insert, also tracked with the ANY-maze®
1048 software and the AMi-maze® interface to register the track of the mice. Mice were
1049 accustomed to this environment for 10 min during two consecutive days, and the test
1050 was performed on the third day. Mice were left to explore two identical equidistant
1051 cubes for 5 min (the familiarization phase) and returned for 15 min into its cage. One
1052 cube was substituted for a similar size and color sphere and mice were returned to the
1053 arena to explore the objects for other 5 min (the test phase). To score zone entries that
1054 consider the exploration of an object we consider the size of the object (3.8x3.8 cm) and
1055 the surrounding perimeter (6x6cm). The ability to recognize the sphere as a novel object
1056 was determined as discrimination index (DI) calculated as $[DI = (T_N - T_F) / (T_N + T_F)]$,
1057 where T_N is the time spent exploring the new object (sphere) and T_F is the time spent
1058 exploring the familiar object (cube). The number of evaluated animals per test was
1059 specified when corresponding ($8 \geq n \leq 13$).

1060

1061 **Statistical analysis.** Immunohistochemistry quantifications were performed in brain
1062 samples from three male mice (n=3). For experiments involving behavior and those
1063 requiring *in vivo* or *ex vivo* samples, male mice were used and the number and ages of
1064 animals per condition are specified where appropriate. *In vitro* experiments were
1065 evaluated with 3-9 independent biological replicates obtained from 2-4 independent
1066 culture preparations. Typically, 4-6 technical replicates were carried out per biological
1067 replicate. Data were expressed as mean \pm standard error of the mean (SEM) values
1068 using as “n” the number of independent culture preparations. The statistical
1069 comparisons between two groups of values were performed using the two-tailed
1070 Student’s *t*-test. In all cases, $p < 0.05$ values were considered significant. Statistics were
1071 performed using Microsoft Excel or the IBM SPSS Statistics software.

1072

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