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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 process<sup>1</sup> that requires the  
35 metabolic cooperation of different cell types<sup>2-4</sup>. Astrocytic-derived lactate sustains  
36 memory consolidation<sup>5</sup> by serving as an energetic oxidizable substrate for neurons<sup>6</sup> and  
37 a signaling molecule<sup>7,8</sup>. Factors that support this metabolic compartmentalization are  
38 differential regulation of glycolytic enzymes<sup>9</sup> and mitochondrial respiratory chain  
39 organization<sup>10</sup> between neurons and astrocytes. Thus, unlike neurons, astrocytes rely  
40 heavily on glycolysis for energy generation<sup>9</sup> and, as a consequence, have a loosely  
41 assembled mitochondrial respiratory chain that allows a higher generation of  
42 mitochondrial reactive oxygen species (ROS)<sup>10</sup>. 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 activity<sup>11</sup>. A  
60 floxed (LoxP-flanked) transcriptional STOP cassette was incorporated between the  
61 mitochondrial-tagged catalase cDNA and the CAG promoter (mCAT<sup>LoxP/+</sup>), in order to  
62 eventually obtain tissue- and time-specific expression of mitochondrial-tagged catalase  
63 *in vivo* (Figure 1a). mCAT<sup>LoxP/+</sup> 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 H<sub>2</sub>O<sub>2</sub> (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<sup>LoxP/+</sup>  
77 mice was mated with mice, under the C57BL/6-J background, harboring the estrogen  
78 receptor (ERT2)-inducible (tamoxifen-dependent) Cre recombinase (Cre<sup>ERT2</sup>) governed  
79 by the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter (GFAP-  
80 Cre<sup>ERT2/+</sup>)<sup>12</sup> (Figure 1a). The progeny, namely GFAP-Cre<sup>ERT2/+</sup>; mCAT<sup>LoxP/+</sup>, and +/+;  
81 mCAT<sup>LoxP/+</sup> 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<sup>ERT2/+</sup>; mCAT/+ (henceforth GFAP-mCAT) and +/+; mCAT<sup>LoxP/+</sup>  
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 H<sub>2</sub>O<sub>2</sub> 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 PPP<sup>13</sup>. G6PD is transcriptionally regulated<sup>14</sup> 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 expression<sup>15</sup>, was higher in mCAT astrocytes (Figure 2e and Figure  
123 S4b) indicating the predominance of the reduced (active) form of HDAC4 in these  
124 cells<sup>16</sup>. 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 <sup>14</sup>CO<sub>2</sub> production  
130 from mCAT astrocytes incubated in the presence of [1-<sup>14</sup>C]glucose, which is  
131 decarboxylated in the PPP and in the tricarboxylic acid (TCA) cycle<sup>17</sup>. As shown in  
132 Figure 2g (left panel), <sup>14</sup>CO<sub>2</sub> production from [1-<sup>14</sup>C]glucose significantly increased in  
133 mCAT when compared with control astrocytes; however, the rate of [6-<sup>14</sup>C]glucose  
134 oxidation, which takes place exclusively in the TCA cycle<sup>17</sup> was unaffected (Figure 2g,

135 middle panel). Thus, the difference between the rates of oxidation from [1-<sup>14</sup>C]- and [6-  
136 <sup>14</sup>C]glucose, which reflects the rate of glucose oxidation *via* PPP<sup>9</sup>, 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-<sup>3</sup>H]glucose into <sup>3</sup>H<sub>2</sub>O (Figure 2i). These results are in good  
142 agreement with the notion that PPP and glycolysis are mutually affected pathways in  
143 several experimental paradigms<sup>9</sup>. 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<sup>+</sup>)/NADP<sup>+</sup> ratio<sup>1,13</sup>, we then aimed  
148 to ascertain whether mROS physiologically influences the redox status of this  
149 nicotinamide dinucleotide. The NADPH(H<sup>+</sup>)/NADP<sup>+</sup> ratio was significantly lower in  
150 the mCAT when compared with WT astrocytes (Figure 3a), a result compatible with  
151 increased NADPH(H<sup>+</sup>) consumption. NADPH(H<sup>+</sup>) oxidases (NOXs) are active  
152 NADPH(H<sup>+</sup>) oxidizing enzymes<sup>18</sup>, and we previously reported that NOX-1 and -2 are  
153 largely the most abundant NOXs isoforms present in astrocytes<sup>10</sup>. 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 (O<sub>2</sub><sup>•-</sup>) abundance. In good agreement  
158 with the observed enhanced expression of NOX-1 and -2, we found higher extracellular  
159 O<sub>2</sub><sup>•-</sup> 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)<sup>19,20</sup>, 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 activity<sup>21</sup>, 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)<sup>22</sup> 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<sup>1,5,7</sup>, 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<sup>22,23</sup>, 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 biosynthesis<sup>24</sup>. 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 decline<sup>25</sup>, 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 H<sub>2</sub>O<sub>2</sub> unspecificity<sup>26</sup>. 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<sup>1,9,27</sup>. 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 balance<sup>22</sup>. 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 stress<sup>28</sup>. Accordingly,  
263 antioxidant compounds administration has shown protection in pre-clinical models of  
264 neurological conditions closely associated with redox stress<sup>29,30</sup>. Unfortunately,  
265 translation of this knowledge into clinical trials have failed so far<sup>29</sup>, 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 therapy<sup>29</sup>. 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*<sup>31</sup> and mice<sup>32</sup>. In sum, an increasing body of

271 evidence now suggests that ROS exert physiological functions in several tissues<sup>33</sup>;  
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 neurons<sup>10</sup>. As by analogy  
276 established for exercise<sup>34</sup>, cancer growth<sup>35</sup> and general mortality<sup>36</sup>, 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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291

### 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  $\mu$ m.

414 (e) Isolated mitochondria from astrocytes in primary culture obtained from mCAT mice  
415 detoxify exogenously-added H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub>, as determined both in isolated mitochondria and in whole cells.  
419 Absolute data were 2.6 or 16.7 pmol/h x  $\mu$ 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<sup>+</sup> cells) from other neural cells (ACSA-2<sup>-</sup>) 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  $\mu\text{m}$ ), as assessed  
431 in the acutely isolated ACSA-2<sup>+</sup> and ACSA-2<sup>-</sup> cell fractions.

432 (j) *Ex vivo* analysis of H<sub>2</sub>O<sub>2</sub> production in the acutely isolated ACSA-2<sup>+</sup> cell fraction.  
433 Absolute data were 1.7 pmol/h x  $\mu\text{g}$  protein in control ACSA-2<sup>+</sup> 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 ( $\Delta\Psi_{\text{m}}$ ) 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 <sup>14</sup>CO<sub>2</sub> production from [1-<sup>14</sup>C]glucose (left) and [6-<sup>14</sup>C]glucose (middle) in  
460 WT and mCAT astrocytes. The right panel shows the difference between the rates of  
461 <sup>14</sup>CO<sub>2</sub> production from [1-<sup>14</sup>C]- minus that from [6-<sup>14</sup>C]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<sup>+</sup>)**  
472 **oxidase-mediated extracellular superoxide in mCAT astrocytes and leads to**  
473 **neuronal damage.**

474 (a) The NADPH(H<sup>+</sup>)/NADP<sup>+</sup> 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<sup>+</sup>) 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 (O<sub>2</sub><sup>•-</sup>) 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 (O<sub>2</sub><sup>•-</sup>)  
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 H<sub>2</sub>O<sub>2</sub> 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. H<sub>2</sub>O<sub>2</sub> absolute values were 0.6 pmol/h x  $\mu$ 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  $\mu$ 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<sup>LoxP/+</sup>), in order to eventually  
557 obtain tissue- and time-specific expression of mitochondrial-tagged catalase *in vivo*.  
558 mCAT<sup>LoxP/+</sup> 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<sup>LoxP/+</sup> mice was mated with mice, under a  
564 C57BL/6-J background, harboring the estrogen receptor (ERT2)-inducible (tamoxifen-  
565 dependent) Cre recombinase (Cre<sup>ERT2</sup>) governed by the astrocyte-specific glial fibrillary  
566 acidic protein (GFAP) promoter (GFAP-Cre<sup>ERT2/+</sup>)<sup>1</sup>. The progeny, namely GFAP-  
567 Cre<sup>ERT2/+</sup>; mCAT<sup>LoxP/+</sup>, and +/+; mCAT<sup>LoxP/+</sup> 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<sup>ERT2/+</sup>; mCAT/+ (henceforth GFAP-mCAT) and +/+; mCAT<sup>LoxP/+</sup> 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'-GTAGGGCCAACTGTTTCTGCATGA-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 allele<sup>2</sup> (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 neonates<sup>3</sup>. Cell suspension were seeded  
604 in 75 cm<sup>2</sup> 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 10<sup>5</sup> cells/cm<sup>2</sup> 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  $2.0 \times 10^5$   
617 cells/cm<sup>2</sup> 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% CO<sub>2</sub>-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<sup>®</sup> 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 construct<sup>3</sup>. Cells were used after 72 hours of transfection. To overexpress miR-  
632 206, 3 nM of Ambion Pre-miR<sup>TM</sup> miRNA precursor for miR-206 (PM10409, Cat.  
633 #AM17100) or the Ambion Pre-miR<sup>TM</sup> 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 protocol<sup>4</sup>. 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 protocol<sup>5</sup> based on the ability of  
650 catalase to eliminate exogenous H<sub>2</sub>O<sub>2</sub>. 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 H<sub>2</sub>O<sub>2</sub> for 0, 5, 10, 15 and 20 minutes. Reactions were stopped by adding HCl  
653 (37% final concentration, vol/vol). Afterwards, 3.2 mM (NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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% CO<sub>2</sub> atmosphere in HBSS buffer (NaCl 134.2 mM; KCl 5.26 mM;  
663 KH<sub>2</sub>PO<sub>4</sub> 0.43 mM; NaHCO<sub>3</sub> 4.09 mM; Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O 0.33 mM; glucose 5.44 mM;  
664 HEPES 20 mM; CaCl<sub>2</sub>·2H<sub>2</sub>O 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 **H<sub>2</sub>O<sub>2</sub> determination.** For H<sub>2</sub>O<sub>2</sub> assessments, Amplex™ Red Hydrogen  
670 Peroxide/Peroxidase Assay Kit (Life Technologies) was used. Cultured astrocytes or  
671 ACSA-2<sup>+</sup> 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; Na<sub>2</sub>HPO<sub>4</sub> 5.7 mM; KCl 4.86 mM; CaCl<sub>2</sub> 0.54 mM; MgSO<sub>4</sub> 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) ( $\lambda_{exc}$  = 538 nm;  $\lambda_{em}$  = 604 nm). Slopes

677 were used for the calculations of the rates of H<sub>2</sub>O<sub>2</sub> formation, and the results were  
678 expressed as fold change.

679

680 **Extracellular superoxide (O<sub>2</sub><sup>•-</sup>) determination.** The production of extracellular O<sub>2</sub><sup>•-</sup>  
681 was measured following a previous published protocol<sup>6</sup>. 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 O<sub>2</sub><sup>•-</sup> 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 previously<sup>7</sup>. Total glutathione was measured in reaction buffer (0.1 mM NaHPO<sub>4</sub>, 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 ( $\lambda_{\text{exc}}=360$  nm,  $\lambda_{\text{em}}=460$  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 cm<sup>2</sup> flasks of  
717 adherent cells at 60-70% confluence containing a central microcentrifuge tube with  
718 either 0.8 ml benzethonium hydroxide (Sigma) for <sup>14</sup>CO<sub>2</sub> equilibration or 1 ml H<sub>2</sub>O for  
719 <sup>3</sup>H<sub>2</sub>O equilibration. Incubations were carried out in KRPG containing 5 mM D-glucose  
720 at 37 °C in the air-thermostatized 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 <sup>14</sup>C]glucose or [6-<sup>14</sup>C]glucose for 90 min as previously described<sup>8,9</sup>. Incubations were  
726 then terminated by the addition of 0.2 ml 20% perchloric acid (Merck Millipore) for 40  
727 minu before the benzethonium hydroxide (containing <sup>14</sup>CO<sub>2</sub>) 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 <sup>14</sup>CO<sub>2</sub> production from  
730 [1-<sup>14</sup>C]glucose (which decarboxylates through the 6-phosphogluconate dehydrogenase-  
731 catalyzed reaction) and that of [6-<sup>14</sup>C]glucose (which decarboxylates through the  
732 tricarboxylic acid cycle)<sup>8,10</sup>. Glycolytic flux was measured by assaying the rate of <sup>3</sup>H<sub>2</sub>O  
733 production from [3-<sup>3</sup>H]glucose using a similar method but instead incubating cells with  
734 5 μCi D-[3-<sup>3</sup>H]glucose in KRPG buffer per flask for 120 minutes as previously  
735 described<sup>8,9</sup>. Incubations were then terminated with 0.2 ml 20% perchloric acid, and  
736 cells further incubated for 96 hours with a microcentrifuge tube, containing H<sub>2</sub>O,  
737 suspended above the cells to allow <sup>3</sup>H<sub>2</sub>O equilibration. The <sup>3</sup>H<sub>2</sub>O was then measured by  
738 liquid scintillation counting (Tri-Carb 4810 TR, PerkinElmer). Under these  
739 experimental conditions, 75% of the produced <sup>14</sup>CO<sub>2</sub> or 28% of the produced <sup>3</sup>H<sub>2</sub>O were  
740 recovered, which were used for the calculations as previously established<sup>9</sup>.

741

742 **Lactate determination.** Lactate concentrations were measured in the culture medium  
743 spectrophotometrically<sup>8</sup> by determining the increments in absorbance of the samples at



744 340 nm in a mixture containing 1 mM NAD<sup>+</sup> 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<sup>+</sup> ratio determination.** This was performed using a colorimetric  
748 NADP<sup>+</sup>/NADPH assay kit (Abcam). Cells were re-suspended in 500 µl of  
749 NADP<sup>+</sup>/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<sup>+</sup>  
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<sup>+</sup>. The actual NADP<sup>+</sup> and NADPH<sup>+</sup>  
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™ DiIC<sub>1</sub>(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 capsid<sup>11</sup> 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-ABC<sub>1</sub>D-GFP)<sup>12</sup>. 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 O<sub>2</sub> and NO<sub>2</sub> (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 10<sup>10</sup> VG (viral genomes) of  
773 AAV-PHP.eB-gfa-ABC<sub>1</sub>D-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 ones<sup>13</sup>. 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 resuspension<sup>14</sup>. The cell suspension was  
779 incubated with MitoSox<sup>TM</sup> and MitoProbe<sup>TM</sup> DiIC<sub>1</sub>(5), as described above, to assess  
780 mitochondrial ROS and mitochondrial membrane potential, respectively, in GFP<sup>+</sup> 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 markers<sup>14</sup>.

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 Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0), supplemented with phosphatase (1 mM Na<sub>3</sub>VO<sub>4</sub>, 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 protocol<sup>3</sup> was used, with some modifications.  
803 This method first uses a mildly aggressive solution (cytosolic buffer; 10 mM HEPES,  
804 1.5 mM MgCl<sub>2</sub>, 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 MgCl<sub>2</sub>, 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 used<sup>15</sup>. Cells grown in 145 cm<sup>2</sup> 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- $\beta$ -ACTIN (1/30000) (A5441, Sigma).

831

832 **Western blotting.** Cells were lysed in RIPA buffer (1% SDS; 2 mM EDTA; 12.5 mM  
833 Na<sub>2</sub>HPO<sub>4</sub>; 1% triton X-100; 150 mM NaCl; pH 7), supplemented with phosphatase (1  
834 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF) and protease (100  $\mu$ M phenylmethylsulfonyl fluoride  
835 (PMSF), 50  $\mu$ g/ml aprotinine, 50  $\mu$ g/ml leupeptine, 50  $\mu$ g/ml pepstatin, 50  $\mu$ g/ml anti-  
836 papain, 50  $\mu$ g/ml amastatin, 50  $\mu$ g/ml bestatin and 50  $\mu$ g/ml soybean trypsin inhibitor)  
837 inhibitor cocktail, 100  $\mu$ M phenylmethylsulfonyl fluoride and phosphatase inhibitors (1  
838 mM o-vanadate). Samples were boiled for 5 min. Aliquots of cell lysates (50  $\mu$ 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 analysis<sup>3</sup>.

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'-GGTGTGGACCTTGCAAAATG-

879 3' and 5'- GGCAGTCATCCACATCCACTT-3' (*Trx1*). The mRNA abundance of each  
880 transcript was normalized to the  $\beta$ -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  $\mu$ l  
909 of 100 nM dNTP, 0.14  $\mu$ l of RNase inhibitor (20 U/ $\mu$ l), 0.67  $\mu$ l of reverse transcriptase  
910 (50 U/ $\mu$ l), 1  $\mu$ l RT-Buffer 1X and 4.5  $\mu$ l of primer pool in a final volume of 10  $\mu$ 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- $\mu$ 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  $\mu$ 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 (Menzel<sup>TM</sup>, Thermo)<sup>16</sup>.

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  $\infty$ /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)<sup>16</sup>.

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.

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