

1 **AIM2 Inflammasome Mediates Hallmark Neuropathological Alterations**
2 **and Cognitive Impairment in a Mouse Model of Vascular Dementia**

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41 **Abstract**

42 Chronic cerebral hypoperfusion is associated with vascular dementia (VaD).
43 Cerebral hypoperfusion may initiate complex molecular and cellular inflammatory
44 pathways that contribute to long-term cognitive impairment and memory loss. Here
45 we used a bilateral common carotid artery stenosis (BCAS) mouse model of VaD to
46 investigate its effect on the innate immune response – particularly the inflammasome
47 signaling pathway. Comprehensive analyses revealed that chronic cerebral
48 hypoperfusion induces a complex temporal expression and activation of
49 inflammasome components and their downstream products (IL-1 β and IL-18) in
50 different brain regions, and promotes activation of apoptotic and pyroptotic cell death
51 pathways. Polarized glial cell activation, white matter lesion formation and
52 hippocampal neuronal loss also occurred in a spatiotemporal manner. Moreover, in
53 AIM2 knockout mice we observed attenuated inflammasome-mediated production of
54 proinflammatory cytokines, apoptosis and pyroptosis, as well as resistance to chronic
55 microglial activation, myelin breakdown, hippocampal neuronal loss, and behavioural
56 and cognitive deficits following BCAS. Hence, we have demonstrated that activation
57 of the AIM2 inflammasome substantially contributes to the pathophysiology of chronic
58 cerebral hypoperfusion-induced brain injury and may therefore represent a promising
59 therapeutic target for attenuating cognitive impairment in VaD.

60

61

62 **Introduction**

63 Vascular dementia (VaD) is the second most common form of dementia that is
64 associated with vascular diseases resulting in dysfunction and damage to the
65 cerebral vasculature, which causes disruption of cerebral blood flow, brain injury, and
66 ultimately cognitive impairment and memory loss¹. Clinical findings in VaD include
67 chronic cerebral hypoperfusion, white matter lesions (WMLs), strokes, hippocampal
68 atrophy, decline in executive functioning and memory impairment². Recent evidence
69 indicates that chronic cerebral hypoperfusion is associated with systemic
70 inflammation before WMLs and neurological symptoms develop in VaD³⁻⁵. Interleukin
71 1-beta (IL-1 β), a potent cytokine that orchestrates inflammatory pathways, is likely to
72 be a key contributor to VaD⁵. The primary molecular machinery for IL-1 β production
73 in response involves assembly and activation of the inflammasome.

74

75 Inflammasome is an intracellular multi-protein complex that initiates an innate
76 immune response, and is involved in multiple acute and chronic neurological
77 diseases such as ischemic stroke, Alzheimer's disease (AD), Parkinson's Disease
78 (PD), and amyotrophic lateral sclerosis (ALS)⁶⁻¹¹. In the canonical inflammasome
79 pathway, stimulation and homo-oligomerization of inflammasome receptors such as
80 nucleotide-binding domain and leucine-rich repeat containing (NLR) family pyrin
81 domain containing (NLRP) 1 (NLRP1), NLRP3, NLR family CARD domain containing
82 4 (NLRC4) and absent in melanoma 2 (AIM2) initiates formation of the
83 inflammasome complex, recruitment and activation of adaptor (ASC) and effector
84 (total caspase-1 and -8) inflammasome components into biologically active cleaved
85 caspases-1 and -8^{6,12}. In the noncanonical inflammasome pathway, caspase-11 is
86 activated via auto-proteolysis upon dimerization to produce cleaved caspase-11.
87 Upon inflammasome assembly and activation, activity of cleaved caspase-1 and -8
88 are increased and downstream proinflammatory cytokines such as mature IL-1 β and
89 IL-18 are generated⁶. This leads to activation of glial cells, endothelial dysfunction,
90 and oligodendrocyte injury and demyelination, eventually resulting in neurovascular
91 unit disintegration, neuronal loss and brain circuit dysfunction. Cleaved caspase-1
92 and -8 may also promote cell death by activating apoptosis and pyroptosis
93 pathways¹³⁻¹⁴. Cleaved caspase-1 and -8 induces apoptosis and pyroptosis by
94 catalyzing the cleavage of total caspase-3 and full-length Gasdermin D (GSDMD-FL)
95 into cleaved caspase-3 and N-terminal Gasdermin D (GSDMD-NT), respectively.
96 GSDMD-NT can oligomerize with other N-terminal units and cause pore formation in
97 the nuclear and plasma membranes, leading to leakage of cellular content and
98 proinflammatory cytokines^{15,16}. GSDMD activation can also be facilitated directly by
99 non-canonical cleaved caspase-11¹⁷. The inhibition of caspase-1 was demonstrated
100 to prevent pyroptosis in experimental models of multiple sclerosis by reducing
101 demyelination and neurodegeneration¹⁸. It was also established that the
102 inflammasome complex also contributed to cognitive decline in a APP/PS1 mouse
103 model of Alzheimer's disease^{9,19}. Specifically, it was shown that deletion of the AIM2
104 inflammasome in that AD model promoted dendrite branching and synaptic plasticity,
105 and improvement in spatial memory²⁰. AIM2 is known to play a role in the host
106 immune response to microbial DNA²¹; however, several reports have established that
107 endogenous double-stranded DNA (dsDNA) can also activate AIM2 to form the AIM2

108 inflammasome^{22,23}. Moreover, AIM2 also responds to dsDNA released from damaged
109 host cells and is a potential ligand that can activate AIM2 in neurons and glial cells
110 following chronic hypoperfusion^{22, 24}.

111

112 While evidence for direct involvement of the inflammasome complex in VaD is
113 lacking, a cytokine profile of plasma from VaD patients found IL-1 β to be the most
114 abundant²⁵⁻²⁸. It was also reported that IL-1 β impeded oligodendrocyte recruitment
115 and inhibited white matter repair and functional recovery during the early stages of
116 chronic cerebral hypoperfusion⁵. However, any potential involvement of
117 inflammasome activation in chronic cerebral hypoperfusion-induced glial activation,
118 white matter damage, neuronal cell death and cognitive dysfunction has not been
119 studied.

120

121 In the present study, we have comprehensively investigated the role of the
122 inflammasome signaling pathway in the bilateral common carotid artery stenosis
123 (BCAS) mouse model of VaD. The BCAS mouse model has been well established in
124 imitating a wide range of neurological clinical conditions of white matter rarefaction,
125 hippocampal atrophy and cognitive decline induced by chronic cerebral
126 hypoperfusion in humans^{29,30}. Moreover, BCAS develops white matter lesions without
127 cerebral infarctions and optic nerve damage, while hippocampal neuronal death and
128 atrophy is induced making it the most appropriate animal model in studying VaD³¹.
129 Using the BCAS mouse model, we report an increased expression and activation of
130 inflammasome complexes, production of mature proinflammatory cytokines,
131 polarized glial activation, WMLs, and neuronal cell death occurring in a time-
132 dependent manner in the cerebral cortex, hippocampus and striatum following BCAS.
133 Notably, these brain regions exhibited distinct profiles of inflammasome expression
134 and activation, and a specific prominent increase of AIM2 in the cerebral cortex and
135 hippocampus. Furthermore, we demonstrated that genetic deletion of AIM2 protects
136 against BCAS-induced inflammasome activation, proinflammatory cytokine
137 production, polarized glial activation, WML formation and neuronal cell death,
138 ultimately leading to an improvement in cognitive outcome. Overall, our findings
139 reveal that the AIM2 inflammasome may be a potential therapeutic target in VaD.

140

141

142 **Materials and Methods**

143 **Experimental Animals and Genotyping**

144 All *in vivo* experimental procedures were approved by the National University of
145 Singapore, Singapore Animal Care and Use Committee and performed according to
146 the guidelines set forth by the National Advisory Committee for Laboratory Animal
147 Research (NACLAR), Singapore. Mice were housed in individual cages under
148 standard laboratory conditions. All efforts were made to minimize suffering and
149 numbers of animals used. All sections of the manuscript were performed and
150 reported in accordance with ARRIVE (Animal Research: Reporting In Vivo
151 Experiments) guidelines. Fourteen to sixteen weeks old wild type (WT) male
152 C57BL/6 mice (weighing 24 to 30 grams) were obtained from In Vivos, Singapore.
153 The AIM2 knockout (B6.129P2-Aim2^{<Gt(CSG445)Byg>/J}; AIM2 KO) mice were
154 generated by Ingenious Targeting Laboratory (Ronkonkoma, NY) on the C57BL/6
155 background mice via targeted replacement of the AIM2 coding region with a
156 neomycin resistance gene through homologous recombination that was kindly
157 obtained from our collaborator Professor Jenny P. Y. Ting³². Primer sequences used
158 for PCR-based AIM2 deficient mouse genotyping are as follows: neomycin (KO)
159 forward, GGA ACTTCGCTAGACTAGTACGCGTG; neomycin (KO) reverse,
160 CAACATTGTACAGATTGAGCAGG; AIM2 (WT) forward,
161 GATGGAGAGTGAGTACCGGGAAATGCTGTT; and AIM2 (WT) reverse,
162 TCTGCAAGTAGATTGGAGACAGACTCTGGTGA, resulting in a 450-bp band for the
163 wild type and a 250-bp band for the targeted AIM2 knockout when PCR analysis was
164 performed on the genomic DNA (Supplementary Fig. 1). All mice were given free
165 access to food and water *ad libitum*. The experimental groups consisted of 20-25
166 animals in each group.

167

168 **Bilateral Common Carotid Artery Stenosis (BCAS) Mouse Model**

169 Animals were anaesthetized with isoflurane and subjected to BCAS, and
170 subsequent chronic hypoperfusion using microcoils specifically designed for the
171 mouse (microcoil specifications: piano wire diameter 0.08mm, internal diameter
172 0.18mm, coiling pitch 0.5mm, and total length 2.5mm; Sawane Spring Co Ltd, Japan)
173 was described elsewhere³³. The left and right common carotid arteries (CCAs) were
174 exposed individually, freed from their sheaths, and a microcoil was twined by rotating

175 it around each CCA. The site of surgery was closed, and the mice were observed
176 and taken care of post-surgery until conscious and recovered to freely access food
177 and water ad libitum. To study the disease progression, the experimental animals
178 were divided into eight time-point groups: Sham, 1, 3, 7, 15, 21, 30 and 60-days
179 BCAS. Sham animals were given a skin incision and their CCAs were exposed. All
180 animals were euthanized at their respective end-point after BCAS for subsequent
181 analysis. For biochemical analysis involving Wild Type control and AIM2 KO mice,
182 the animals were divided into three experimental groups: Sham, 15 and 30-days
183 BCAS. However, only the Sham and 30-days timepoints were selected to challenge
184 the cognitive ability of both WT and AIM2 KO mice during behavioral analysis.

185

186 **Measurements of Cerebral Blood Flow by Laser Speckle Contrast Imager**

187 High-resolution Laser Speckle Contrast Imager (PSI system, Perimed Inc.) was
188 used to image cerebral blood perfusion and record cerebral blood flow (CBF) before
189 BCAS (baseline), immediately after BCAS surgery and finally at their respective end-
190 points of BCAS. As shown in Supplementary Fig. 2, regions of interest (ROIs)
191 between the bregma and lambda were selected for overall perfusion in the area of
192 two hemispheres. Body temperature was maintained at 37 ± 0.5 °C, and the skull
193 was shaved and exposed by a midline skin incision. The skull was cleaned gently
194 with sterile phosphate buffered saline (PBS) using a cotton applicator. Finally, the
195 image area was kept moist and a non-toxic silicon oil was applied on the skull, which
196 improved imaging. Perfusion images were acquired using the PSI system with a
197 70mW built-in laser diode for illumination and a 1388 x 1038 pixels CCD camera
198 installed 10cm above the skull (speed 19Hz, and exposure time 6mSec). Acquired
199 images were analyzed for changes in CBF (cerebral perfusion) using a dedicated
200 PIMSsoft program (Perimed Inc.).

201

202 **Sample Collection and Processing**

203 Following each time points, mice were euthanized by administering a lethal dose
204 of inhaled carbon dioxide (CO₂) and the brains harvested. The different brain areas
205 (cerebral cortex, hippocampus and striatum) were immediately separated on ice and
206 were frozen in dry ice for tissue biochemical analysis (n = 7-8 in each experimental
207 group). A separate group of animals were sacrificed for histological analysis. Mice
208 were deeply anaesthetized, and perfusion fixation through the heart was performed

209 with 25mL of chilled 1xPBS (pH 7.4) then followed by 25mL chilled paraformaldehyde
210 (4%). Once the perfusion was completed, the brains were harvested and placed in
211 vials containing 4% paraformaldehyde solution for immersion-fixation overnight at
212 4°C (n = 5-7 in each experimental group).

213

214 **Immunoblot Analysis**

215 Immunoblot analysis was performed as described by us previously³⁴. Briefly, brain
216 tissues were homogenized in lysis buffer and then combined with 2 x Laemelli buffer
217 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein samples were then
218 separated on 7.5 to 12.5% v/v sodium dodecyl sulfate (SDS) gels. The SDS-PAGE
219 gels were transferred onto nitrocellulose membranes to probe for proteins. Next, the
220 nitrocellulose membranes were incubated with the following primary antibodies:
221 NLRP1 (Santa Cruz, sc390133), NLRP3 (Adipogen, AG20B0014), NLRC4 (Millipore,
222 06-1125), AIM2 (Cell Signaling, #13095), IL-1 β (Genetex, GTX74034), IL-18
223 (Biovision, 5180R-100), Total Caspase-1 and Cleaved Caspase-1 (p33 subunit)
224 (Adipogen, AG20B0042), Cleaved Caspase-1 (p20 subunit) (Cell Signaling, #67314),
225 Casapase-8 (Cell Signaling, #4927), Caspase-11 (Cell Signaling, #14340), Total
226 Caspase-3 (Cell Signaling, #9662), Cleaved Caspase-3 (Cell Signaling, #9664),
227 GSDMD (Cell Signaling, #93709), GSDMD-NT (Cell Signaling, #50928), GSDME
228 (Santa Cruz, sc393162), IBA-1 (Abcam, ab5076), GFAP (Cell Signaling, #12389),
229 and β -actin (Sigma-Aldrich, A5441) overnight at 4°C with agitation. Following primary
230 antibody incubation, membranes were washed three times with 1xTBST before
231 incubating with horseradish peroxidase (HRP)-conjugated secondary antibodies
232 (Goat Anti-Rabbit – Cell Signaling Technology, Danvers, MA, USA; Goat Anti-Mouse
233 – Sigma-Aldrich, St. Louis, MO, USA; Goat Anti-Rat – GE Healthcare Life Sciences,
234 Little Chalfont, UK) for 1hr at 24°C with agitation. Following secondary antibody
235 incubation, membranes were washed three times with 1xTBST, each time for 10 min.
236 The substrate for HRP, enhanced chemiluminescence (ECL) (Bio-Rad Laboratories,
237 Inc., Hercules, CA, USA) was applied before the membranes were imaged using
238 ChemiDocXRS+imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).
239 Quantification of proteins was conducted using Image J software (Version 1.46;
240 National Institute of Health, Bethesda, MD, USA), where protein densitometry was
241 expressed relative to the densitometry of the corresponding β -actin.

242 **Luxol Fast Blue and Cresyl Violet Staining**

243 Mouse brain tissues were fixed in 10% neutral buffered formalin and then
244 processed into paraffin wax blocks. Coronal sections (4µm thick) were obtained via
245 microtome sectioning. Axonal fibre density of Luxol-fast-blue (LFB) staining was
246 performed to detect the severity of white matter (WM) lesions. Briefly, de-waxed
247 rehydrated sections were immersed in the LFB solution (Abcam, UK) at 37°C
248 overnight. Excess staining was removed by 95% ethanol treatment followed by
249 washing with deionized water. Grey and white matter differentiation was initiated with
250 the treatment of 0.05% aqueous lithium carbonate (Abcam, UK) for 20 seconds,
251 followed by 70% ethanol until the nuclei are decolorized. Sections were immersed in
252 Cresyl Violet solution (Abcam, UK) for 5 min and washed in deionized water. The
253 sections were dehydrated in an ethanol gradient (70 – 100%), and finally cleared in
254 xylene and mounted with a mounting agent. The bright field images were taken under
255 4X and 60X magnification using an Olympus upright Fluorescence Microscope BX53.
256 The WM lesions were evaluated in five brain regions: the optic tract, internal capsule,
257 caudoputamen, corpus callosum (Medial) and corpus callosum (Paramedian). The
258 severity of the WM lesions was graded as normal (grade 0), disarrangement of the
259 nerve fibres (grade 1), the formation of marked vacuoles (grade 2), and the
260 disappearance of myelinated fibres (grade 3). The severity of neuropathology was
261 scored by three blinded examiners and the number of neurons in CA1, CA2 and CA3
262 were counted as previously described³⁵. The general morphology and the
263 neurodegeneration in the hippocampus of BCAS animals were assessed by Cresyl
264 violet staining.

265

266 **Immunofluorescence Analysis**

267 Paraffinized brain sections were cut from sham and BCAS animals and
268 immunostained with primary antibodies against Cleaved Caspase-1 (Affinity
269 Biosciences, AF4022), Cleaved Caspase-3 (Cell Signaling, #9661), GSDMD (Santa
270 Cruz, sc393581), MBP (Cell Signaling, #78896), MAP2 (Millipore, MAB3418), GFAP
271 (Cell Signaling, #3670; #12389), IBA-1 (Wako, 016-26721; Cell Signaling, #17198),
272 CD86 (Santa Cruz, sc-28347), CD206 (eBioscience, 12-2069-42), C3 (Santa Cruz,
273 sc-28294), S100S10 (Invitrogen, PA5-95505), OLIG2 (R&D System, AF2418) and
274 PECAM-1 (BD Pharmingen, 553370). Images were captured with an Olympus
275 FluoView FV1000 (Olympus, Japan) laser scanning confocal microscope using

276 20x/0.70 air objective and 100x/1.45 oil objective, with 488nm Argon ion and 543nm
277 HeNe laser as the excitation source. Single confocal images were converted to 512 x
278 512 pixel 12-bit TIFF images.

279

280 **Behavioral Paradigm and Training of BCAS Animals**

281 **Visual Acuity Test:** To assess visual detection, mice were placed on a platform
282 within a rotating drum covered with black and white vertical stripes at consistent
283 spatial frequency^{36, 37}. The automated drum was then rotated in a clockwise direction
284 for 30sec, and subsequently an anti-clockwise direction for 30sec, after a 15sec inter-
285 trial interval. Both larger stripes (2°) and smaller stripes (1°) were tested for all
286 animals before conducting other behavioral tests.

287 **Open Field Test:** Locomotor activity was measured using an open field test³⁸.
288 Each subject was placed in the center of the open field apparatus (20x20x40cm;
289 Clever Sys Inc.; VA, USA). Total distance traveled (cm) and average speed (mm/s)
290 were recorded and tracked via TopScan (Clever Sys Inc.; VA, USA). Data was
291 collected for 35 minutes.

292 **Morris Water Maze:** Morris water maze was performed 3 weeks after surgery
293 (sham or BCAS), as described previously³⁹. Mice were trained 4 times a day at 10-
294 min intervals for 5 consecutive days in this fixed platform training. In each trial, mice
295 were given 60secs to find the platform. Activity of the animal in the water maze was
296 video-tracked by EthoVision software (Noldus Information Technology, Leesburg,
297 VA), and the escape latency was recorded and analyzed. Long term spatial memory
298 was tested on the 6th day, whereby the platform was removed, and each animal was
299 given 60secs in the water maze after being released from four different directions.
300 Time spent around the platform, number of visits to the targeted quadrant and time
301 spent in the targeted quadrant on the probe day were tracked and analyzed. The
302 probe day was designed to fall between 32 to 34 days of BCAS surgery.

303

304 **Double Stranded DNA Measurement**

305 Double stranded DNA from the serum of BCAS animals were measured as
306 previously described using Quant-iT™ PicoGreen® dsDNA Reagent and Kits. Briefly,
307 20µL of plasma sample collected was diluted with 1X TE solution. Subsequently,
308 equal volume of working solution of Quant-iT™ PicoGreen reagent was added. It was
309 then protected from light and incubated for 5 minutes at room temperature. Samples

310 were excited at 485nm and fluorescence intensity was measured at 520nm using a
311 Synergy HT multi-detection microplate reader (BioTek, Winnooski, VT).

312

313 **Statistical Analysis**

314 Experimental data were analyzed by GraphPad Prism 5.02 and 8.0 software
315 (GraphPad Software, San Diego, CA, USA). All values are expressed as mean \pm
316 standard error of the mean (S.E.M). One-way Analysis of Variance (ANOVA) was
317 used, followed by a Bonferroni post-hoc test to determine differences between
318 groups. A P-value <0.05 was deemed to be statistically significant. For behavioral
319 data, non-parametric Kruskal-Wallis test was used to determine differences between
320 groups. A P-value <0.05 was deemed to be statistically significant.

321

322

323 **Results**

324 **Chronic cerebral hypoperfusion increases inflammasome signalling**

325 We first evaluated the protein expression of inflammasome receptors over 60 days
326 of BCAS in the cerebral cortex, hippocampus and striatum (Supplementary Figs. 3, 4
327 & 5). NLRP3, AIM2 and NLRC4 were all increased in the cerebral cortex, while only
328 AIM2 receptor expression was increased in the hippocampus compared to sham
329 controls (Supplementary Fig. 3a-d, 4). NLRP1 expression was increased in the
330 striatum while other inflammasome receptors remained unchanged (Supplementary
331 Figs. 5a & b). Expression of total caspase-8 and precursor IL-1 β were increased in
332 the cerebral cortex after BCAS, whereas a decrease in precursor IL-18 was evident,
333 and ASC and total caspases-1 and -11 were unchanged (Supplementary Fig. 3e & f).
334 In the hippocampus, expression of total caspases-1, -8 -11 and precursor IL-18
335 increased, whereas ASC was decreased after BCAS (Supplementary Fig. 3g & h).
336 ASC, total caspase-8 and precursors for IL-1 β and IL-18 were also increased in the
337 striatum following BCAS (Supplementary Fig. 5c & d).

338

339 To assess canonical inflammasome activation, expression of cleaved caspase-1
340 subunits (p33, transient active unit; p20, final by-product) and -8 were examined.
341 After BCAS, elevated expression of cleaved caspase-1 (p20) and -8 was detected in
342 the cerebral cortex (Fig. 1a & b) and also hippocampus (Fig. 1c & d). Mature IL-1 β

343 and IL-18 – direct downstream markers of inflammasome activation – were also
344 higher in the cerebral cortex and hippocampus after BCAS versus sham controls
345 (Fig. 1a-d). Expression of non-canonical cleaved caspase-11 was also elevated in
346 the cerebral cortex and hippocampus after BCAS (Fig. 1a-d), whereas only cleaved
347 caspase-8, IL-1 β and IL-18 were increased in the striatum (Supplementary Fig. 6).
348 Overall, the data reveal that inflammasome activation occurs in the brain in a spatial-
349 temporal manner as a result of chronic cerebral hypoperfusion.

350

351 **Chronic cerebral hypoperfusion promotes apoptosis and pyroptosis**

352 We next examined cell death mechanisms associated with inflammasome
353 activation. Thus, we assessed the expression of cleaved caspase-3 and GSDMD-NT,
354 along with their precursor proteins after BCAS (Fig. 1e-h; Supplementary Fig. 7 & 8).
355 Our data showed that in the cerebral cortex (Fig. 1e & f) and hippocampus (Fig. 1g &
356 h) the expression of cleaved caspase-3 and GSDMD-NT were increased following
357 BCAS compared to sham controls. Pyroptotic cell death (i.e. GSDMD-NT expression)
358 was more prominent in the cerebral cortex following BCAS (Fig. 1e & f), whereas
359 only apoptosis (i.e. increased cleaved caspase-3 expression) was detected in the
360 striatum following BCAS (Supplementary Fig. 8).

361

362 We also assessed inflammasome-associated secondary pyroptosis/necrosis,
363 which can be induced by cleaved caspase-3, as indicated by expression of N-
364 Terminal Gasdermin E (GSDME-NT). Similar to GSDMD-NT, inflammasome-
365 generated GSDME-NT can permeabilize nuclear and plasma membranes and
366 mitochondria, linking inflammasome activation to cell death¹⁶. We found GSDME-NT
367 to be increased in the cerebral cortex, but not in the hippocampus or striatum
368 following BCAS (Supplementary Fig. 7 & 8). Expression patterns of inflammasome-
369 related proteins and pathways following BCAS-induced chronic cerebral
370 hypoperfusion are summarized in Supplementary Fig. 9. Overall, the data indicate
371 that BCAS induces differential priming of inflammasome components, inflammasome
372 activation, and induction of apoptosis and pyroptosis pathways in the brain in a
373 spatial-temporal manner.

374

375

376 **Chronic cerebral hypoperfusion induces glial cell activation, white matter**
377 **lesions and hippocampal neuronal death**

378 We next analyzed for associations between inflammasome activity and hallmark
379 VaD pathologies involving glial cell activation, white matter integrity and neuronal
380 loss. Expression of ionized calcium binding adaptor molecule 1 (Iba1) and glial
381 fibrillary acidic protein (GFAP) indicated activation of microglia and astrocytes,
382 respectively (Fig. 2a-d). Following BCAS, we found increased Iba-1 in the cerebral
383 cortex (Fig. 2a & b) and increased GFAP in the hippocampus (Fig. 2c & d), and both
384 markers increased in the striatum (Supplementary Fig. 10a & b). Subsequently,
385 colocalization of both Iba1 and GFAP with respective polarization markers CD86,
386 CD206, complement component 3 (C3) and S100A10 revealed activation of mainly
387 M1 (CD86 positive) and M2 (CD206 positive) microglia upon chronic cerebral
388 hypoperfusion in the cerebral cortex and hippocampus (Fig. 2e-h; Supplementary
389 Fig. 11a-h) and striatum (Supplementary Fig. 10c-f). Myelin basic protein (MBP) and
390 Luxol fast blue staining revealed myelin integrity, while cresyl violet and microtubule-
391 associated protein 2 (MAP2) staining enabled assessment of neuronal loss in the
392 hippocampus. Staining for MBP and MAP2 was reduced in the cerebral cortex (Fig.
393 2i & k), hippocampus (Fig. 2j & l) and striatum (Supplementary Fig. 10g & h) following
394 30 days of BCAS.

395

396 White matter integrity of five brain regions was assessed after 7-60 days of
397 hypoperfusion, and found to be disrupted in the corpus callosum (Paramedian and
398 Medial), caudoputamen, internal capsule and optic tract (Fig. 2m). All five areas
399 exhibited time-dependent rarefaction of white matter (Fig. 2n) from 7 days, although
400 the white matter lesion (WML) index was not increased in the optic tract until 60 days
401 of BCAS (Fig. 2m & n). BCAS-induced neuronal loss was evident in hippocampal
402 CA1, CA2 and CA3 areas (Fig. 2o-r). Whereas hippocampal sections from sham
403 controls showed normal neuronal cell bodies with distinct nuclei, nucleoli, and
404 densely packed neurons in all three hippocampal areas, there were signs of
405 widespread neuronal loss evident after BCAS (Fig. 2o-r). Degeneration was more
406 pronounced in the hippocampal CA2 and CA3 areas with severe atrophy from 7 days
407 (Fig. 2o-r).

408

409 **AIM2 activation mediates apoptosis and pyroptosis during chronic cerebral**
410 **hypoperfusion**

411 As AIM2 was upregulated in the cerebral cortex and hippocampus, we postulated
412 its involvement in BCAS-induced brain injury. Analysis of serum revealed an increase
413 in cell-free double stranded DNA (dsDNA) levels following BCAS, especially after 15
414 days (Fig. 3a). As dsDNA is the only known ligand that activates the AIM2 receptor^{22,}
415 ²⁴, the finding supports a potential involvement of the AIM2 inflammasome in BCAS-
416 induced brain injury.

417

418 To further examine the role of AIM2 inflammasome activation on injury following
419 chronic cerebral hypoperfusion, mice with AIM2 deficiency (AIM2 KO) were studied
420 (Supplementary Fig. 1). We first confirmed via laser speckle contrast imaging that
421 cerebral blood flow was equivalent under basal conditions and reduced following
422 BCAS surgery in wild-type (WT) and AIM2 KO mice (Supplementary Fig. 12). This
423 was similar to previously published data from BCAS in mice²⁹. This BCAS-induced
424 reduction in flow was sustained to a similar degree for at least 15 days in both
425 genotypes but was slightly higher in AIM2 KO than WT mice at 30 days
426 (Supplementary Fig. 12a-c).

427

428 We found no difference in the basal expression levels of cleaved caspase-1, -8,
429 and -11, and both mature IL-1 β and IL-18 (Fig. 3b-e) or in the levels of total caspases
430 -1, -8 and -11, or IL-1 β and IL-18 precursors in the cerebral cortex and hippocampus
431 of WT and AIM2 KO mice (Supplementary Fig. 13a-d). However, there was evidence
432 of reduced inflammasome activation following BCAS in AIM2 KO mice when
433 compared to WT control (Fig. 3b-e). Of the three active effector proteins, only
434 canonical cleaved caspase-1 expression was reduced in AIM2 KO mice compared to
435 WT control following BCAS (Fig. 3b-e; Supplementary Fig. 14a & b). There was also
436 reduced levels of mature IL-1 β and IL-18 in the cerebral cortex and hippocampus of
437 AIM2 KO mice at 15 and 30 days following BCAS (Fig. 3b-e; Supplementary Fig. 14a
438 & b). These data thus indicated less inflammasome activity occurs after BCAS in the
439 absence of the AIM2 receptor, especially mediated by canonical cleaved caspase-1.
440 Furthermore, protein expression of both cleaved caspase-3 and GSDMD-NT was
441 lower at 30 days after BCAS in the cerebral cortex (Fig. 3f & g) and hippocampus

442 (Fig. 3h & i) of AIM2 KO mice, indicating AIM2 inflammasome mediating apoptosis
443 and pyroptosis under chronic cerebral hypoperfusion.

444

445 Immunofluorescence studies indicated that cleaved caspase-1 (CC1) activity was
446 lower in AIM2 KO mice in cortical (Fig. 3j; Supplementary Fig. 15a-e & 16a-e) and
447 hippocampal (Fig. 3k; Supplementary Fig. 15f-j & 16f-j) neurons (MAP2 positive) and
448 microglia (Iba-1 positive) compared to WT controls at 30 days following BCAS.
449 Immunoreactivity against cleaved caspase-1 was similar in cortical and hippocampal
450 oligodendrocytes (OLIG2 positive), astrocytes (GFAP positive) and endothelial cells
451 (PECAM-1 positive) of WT and AIM2 KO mice. Cellular specificity of AIM2
452 inflammasome-mediated cell death following BCAS was assessed by
453 immunoreactivity against cleaved caspase-3 (CC3) and GSDMD in the cerebral
454 cortex (Fig. 3j; Supplementary Fig. 15a-e; Supplementary Fig.17a-e & 18a-e) and
455 hippocampus (Fig. 3k; Supplementary Fig. 15f-j; Supplementary Fig.17f-j & 18f-j).
456 The data indicate that pro-apoptotic cleaved caspase-3 immunoreactivity was lower
457 in cortical (Fig. 3j; Supplementary Fig. 15a-e & 17a-e) and hippocampal (Fig. 3k;
458 Supplementary Fig. 15f-j & 17f-j) neurons (MAP2 positive) and microglia (Iba-1
459 positive) of AIM2 KO versus WT mice. Similarly, immunoreactivity of pro-pyroptotic
460 GSDMD was reduced in neurons and microglia in the cortex (Fig. 3j; Supplementary
461 Fig. 15a-e & 18a-e) and hippocampus (Fig. 3k; Supplementary Fig. 15f-j & 18f-j) of
462 AIM2 KO mice. These findings were consistent with more neurons and fewer
463 microglial activation in AIM2 KO than WT mice following chronic cerebral
464 hypoperfusion.

465

466 **AIM2 KO mice are resistant to microglial activation, myelin breakdown,**
467 **hippocampal neuronal loss and cognitive deficits following chronic cerebral**
468 **hypoperfusion**

469

470 There was a lower expression of Iba-1 in the cerebral cortex and hippocampus of
471 AIM2 KO than WT mice after BCAS (Fig. 4a-f), especially in CD86 positive
472 inflammatory microglial cells (Fig. 4c and f and Supplementary Fig. 19a-h). These
473 results suggest a role for AIM2 in microglial activation and polarization during chronic
474 cerebral hypoperfusion. We next investigated if AIM2 KO mice are protected against
475 BCAS-induced myelin injury. Although sham-operated WT and AIM2 KO mice

476 displayed no difference in protein expression and immunoreactivity of MBP, at 30
477 days after BCAS, there was lower expression and weaker immunoreactivity for MBP
478 in the cerebral cortex of WT mice than AIM2 KO mice (Fig. 4g-i). A similar difference
479 in MBP immunoreactivity between WT and AIM2 KO was seen in CA2 and CA3
480 hippocampal regions (Fig. 4j-l). As myelin integrity was healthier in AIM2 KO mice
481 following BCAS, we assessed whether these mice might be resistant to WML. After
482 BCAS, Luxol fast blue staining in WT mice indicated myelin rarefaction and vacuole
483 formation in several WM regions, but a smaller degree of damage was present in the
484 corpus callosum (paramedian or medial), caudoputamen or internal capsule regions
485 of AIM2 KO mice (Fig. 5a-e). Neuronal loss in hippocampal CA1, CA2 and CA3
486 regions was also less in AIM2 KO compared to WT mice at 15 and 30 days of
487 cerebral hypoperfusion (Fig. 5f-h). These data confirm a deleterious role of the AIM2
488 inflammasome in mediating VaD pathologies associated with chronic cerebral
489 hypoperfusion.

490

491 Finally, we tested for a role of the AIM2 inflammasome in BCAS-induced cognitive
492 decline. It was previously shown that BCAS-induced chronic cerebral hypoperfusion
493 in mice resulted in cognitive impairment such as working memory dysfunction and
494 memory loss^{29,30}. To examine the effects of AIM2 KO against BCAS-induced
495 cognitive deficits, we conducted the Morris Water Maze and two other control tests
496 (Fig. 6a-h). To ensure that the animals were able to detect the visual cues during the
497 Morris Water Maze, a visual acuity test was conducted. All animals showed a 100%
498 passing rate, and so proceeded to the subsequent tests. In the open field test
499 conducted to investigate explorative and locomotor ability, WT BCAS mice did not
500 have a shorter distance travelled (Fig. 6a) or a lower average speed of movement
501 (Fig. 6b) when compared to WT Sham controls, indicating no impairment of
502 locomotor ability. Similarly, there was also no significant difference in the time spent
503 and the distance travelled in the outer and inner zone among the WT mice, showing
504 no BCAS-induced anxiety effects (Fig. 6c & d). However, it was observed that both
505 sham and BCAS AIM2 KO mice spent more time at the outer zone as compared to
506 the WT controls (Fig. 6c), indicating that AIM2 KO mice generally exhibit higher
507 levels of anxiety independent of BCAS. Several parameters were assessed in the
508 Morris Water Maze performance, including escape latency during five training days.
509 Generally, we observed a shorter time was taken to reach the platform each day

510 across all groups. Similar daily improvement was exhibited in WT or AIM2 KO mice
511 sham controls and AIM2 KO mice following BCAS, whereas WT mice subjected to
512 BCAS took approximately twice as long (Fig. 6e). Following five days of training,
513 spatial memory was assessed whereby WT mice subjected to BCAS spent less time
514 around the platform and significantly fewer visits to the target quadrant than WT
515 controls (Fig. 6f-h). By contrast, AIM2 KO mice subjected to BCAS spent a longer
516 time around the platform and visited the target quadrant more often than WT BCAS
517 mice (Fig. 6f-h). This suggest that AIM2 KO mice exhibit better memory retention as
518 compared to WT controls under similar BCAS conditions. Thus, the behavioral data
519 indicates that BCAS-induced cognitive impairments were reduced in mice deficient in
520 AIM2, suggesting a pivotal role for the AIM2 inflammasome in mediating VaD
521 pathology associated with chronic cerebral hypoperfusion.

522

523 **Discussion**

524 Our findings indicate that inflammasome receptors are expressed in the cortex and
525 hippocampus where the AIM2 inflammasome is activated in response to chronic
526 cerebral hypoperfusion, leading to cellular pathology and cognitive impairment in a
527 mouse model of vascular dementia. Genetic deficiency of AIM2 expression resulted
528 in less brain inflammation, white matter injury, neuronal loss and cognitive decline
529 during cerebral hypoperfusion. These data are the first to indicate that the AIM2
530 inflammasome promotes neuronal and white matter injury following cerebral
531 hypoperfusion, identifying AIM2 as a key contributor to sterile inflammatory
532 responses in vascular dementia.

533

534 Inflammasomes are intracellular multiprotein complexes composed of sensors for
535 various microbial components, viral RNA and damage- or danger-associated
536 molecular patterns (DAMPs) produced during cell injury^{40,41}. These innate immune
537 complexes are categorized according to their structural characteristics as either
538 nucleotide-binding domain–like receptors (NLRs) or absent in melanoma 2 (AIM2)-
539 like receptors^{41,42}. These receptors oligomerize in response to activation by external
540 stimuli and then recruit the adaptor protein, apoptosis-associated speck-like protein
541 containing a caspase recruitment domain (ASC)⁴³. Ultimately, binding of caspase-1
542 results in its cleavage and activation, and the generation of mature pro-inflammatory

543 cytokines, IL-1 β and IL-18, as well as pro-apoptotic cleaved caspase-3, and pro-
544 pyroptotic N-terminal GSDMD and GSDME⁴⁴. While our study established the role of
545 AIM2 in chronic hypoperfusion-induced brain injury, it is still unknown whether other
546 inflammasomes might have a role in WML formation and neuronal cell death
547 following hypoperfusion. Although our data shows increased expression levels of
548 NLRP3 and NLRC4 in brain tissues following BCAS suggesting a potential
549 involvement of other inflammasome complexes, further studies are required to
550 establish the role of these inflammasomes in chronic hypoperfusion-induced brain
551 injury.

552

553 We previously demonstrated that inflammasomes contribute to neuronal cell death
554 following ischemic stroke⁷, in part via NF- κ B and MAPK signaling³⁴. Our present data
555 indicate that chronic cerebral hypoperfusion may also lead to increased expression
556 and activation of NLRP and AIM2 inflammasome receptors in multiple brain areas,
557 especially in the hippocampal region, which is known to be particularly vulnerable to
558 injury following mild ischemia⁴⁵. Cerebral hypoperfusion in the BCAS model used
559 here is known to impact the hippocampus by mechanisms involving ionic imbalance,
560 excitotoxicity, mitochondrial dysfunction and oxidative stress⁴⁶⁻⁴⁸. I'm not sure what
561 the purpose of this sentence is.

562

563 It is important to elucidate the mechanism(s) by which the AIM2 inflammasome
564 mediates chronic hypoperfusion-induced brain injury. Here we found that the plasma
565 concentration of dsDNA progressively increased following BCAS-induced cerebral
566 hypoperfusion, consistent with the possibility that this ligand promoted AIM2-
567 mediated amplification of brain inflammation and injury. Thus it is plausible that AIM2
568 functions as a DAMP sensor to promote sterile inflammation in vascular dementia.
569 We found that various white matter regions exhibited injury within 7 days of BCAS,
570 consistent with the known vulnerability of white matter to hypoperfusion. Reduced
571 blood flow to the white matter has been shown to predict the clinical development of
572 WMLs within 18 months⁴⁹. We observed that AIM2 deficient animals subjected to
573 BCAS exhibited greater white matter integrity and lower WML severity scores than
574 WT mice after BCAS. Using magnetic resonance imaging, WMLs appear as white
575 matter hyperintensities (WMH) in VaD patients that arises due to demyelination and
576 axonal loss as well as microglial and endothelial activation⁵⁰⁻⁵². In accordance with

577 previous studies, we found that neuronal cell death, loss of myelin basic protein and
578 microglial activation were present in our BCAS mouse model, which were reduced in
579 AIM2 deficient mice. Our data also showed that cerebral blood flow was significantly
580 improved in AIM2 KO mice compared to WT controls following 30 days of BCAS.
581 While we do not know the precise molecular and cellular mechanisms behind this
582 increase, it is possible that reduced inflammasome activation and brain injury may
583 protect the integrity of the neurovasculature to facilitate improved blood flow to the
584 brain.

585

586 The sub-regions of the dorsal hippocampus, CA1, CA2 and CA3, are responsible
587 for memory encoding and retrieval^{53,54}. Our study showed that neurons in CA2 and
588 CA3 hippocampal regions experienced elevated levels of death sooner in
589 comparison to neurons in the CA1 hippocampal regions during chronic hypoperfusion.
590 Reduced levels of BCAS-induced neuronal loss occurred in hippocampal CA2 and
591 CA3 areas of AIM2-deficient animals when compared to WT controls, which is
592 consistent with reduced inflammasome-mediated cell death in the hippocampal
593 region of AIM2 KO mice, suggesting that the AIM2 inflammasome contributed to
594 neuronal loss in the hippocampus during cerebral hypoperfusion.

595

596 The AIM2 receptor is widely expressed in the brain, with its highest levels
597 occurring in microglia under physiological conditions⁵⁵. Furthermore, specifically
598 within the cortex and hippocampus the AIM2 receptor is most abundantly expressed
599 in neurons.⁵⁶ Our data are consistent with a role of AIM2 in these brain resident cells
600 and regions, but we cannot exclude circulating leukocytes as another possible cell
601 mediating the pro-inflammatory actions of AIM2 in the brain in chronic hypoperfusion.
602 Leukocytes contribute to other forms of brain injury and neurodegeneration⁵⁷⁻⁵⁹ and
603 their infiltration from the circulation would be expected in association with reduced
604 blood-brain barrier integrity and blood vessel density known to occur in
605 hypoperfusion-induced VaD⁶⁰. However, in this study we have not assessed the role
606 of leukocyte infiltration and related neutrophil extracellular traps (NETs), known as
607 NETosis, as a potential source of AIM2 ligand. It was previously established that
608 NETosis and its associated extracellular dsDNA is known to contribute to the
609 pathogenesis of number of inflammatory diseases⁶¹. In addition, recent evidences
610 suggest that neutrophils use an inflammasome- and GSDMD-dependent mechanism

611 to activate NETosis, suggesting that further studies are needed to understand the
612 role of NETosis in the pathology of VaD⁶².

613

614 Previous studies have recently reported that the AIM2 inflammasome contributes
615 to brain injury and chronic post-stroke cognitive impairment in mice⁶³, and to reduced
616 neuroplasticity and spatial memory in a mouse model of AD²⁰. Similarly, there is also
617 evidence supporting a role for the NLRP3 inflammasome in cognitive impairment in
618 models of dementia and diabetes^{9,64}. Inflammation is known to adversely impact
619 cognitive function, and our data are consistent with inflammasomes promoting an
620 innate immune response to mediate such an effect during cerebral hypoperfusion.
621 Moreover, we observed in our data that AIM2 KO mice generally exhibited higher
622 levels of anxiety independent of BCAS. This was reported by a previous study that
623 established that AIM2 KO mice exhibited increased anxious behaviors and reduced
624 auditory fear memory at the physiological level⁶⁵.

625

626 In summary, we have provided substantial evidence that activation of the AIM2
627 inflammasome plays a key role in promoting brain inflammation, white matter lesions,
628 neuronal cell death and cognitive impairment induced by chronic cerebral
629 hypoperfusion. These effects of AIM2 involve promotion of apoptosis and pyroptosis
630 of cortical and hippocampal neurons. As inflammasome activation plays a major role
631 in a number of inflammatory diseases, there are substantial efforts to develop
632 inflammasome inhibitors. However, application of inflammasome inhibitors is limited
633 in clinical conditions as clinical trials have not yet been completed. While our study
634 establishes that the AIM2 inflammasome may therefore represent a promising
635 therapeutic target for attenuating cognitive impairment in VaD, further research is
636 warranted to develop specific AIM2 inhibitors to test their effect in experimental and
637 clinical studies.

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646 **Acknowledgements**

647 We thank Professor Jenny P. Ting (University of North Carolina, Chapel Hill, Chapel
648 Hill, NC, USA) for providing the AIM2 deficient mice. Supplementary Figure 1 and
649 Supplementary Figure 9a in this article was created using BioRender.

650

651 **Funding**

652 This work was supported by the National Medical Research Council Research Grants
653 (NMRC-CBRG-0102/2016 and NMRC/OFIRG/0036/2017), Singapore and the startup
654 fund to TVA from La Trobe University, Melbourne, Australia.

655

656 **Author Contributions**

657 Study Conception & Design: T.V.A., L.P., and D.Y.F.; Experiment or Data Collection:
658 T.V.A., L.P., P.W., H.M.L., S.L.F., S.W.K., V.R., S.S., D.Y.F., R.I.V., and N.P.; Data
659 Analysis: L.P., P.W., V.R., S.S., D.Y.F., and T.V.A.; Data Interpretation: L.P., T.V.A.,
660 and D.Y.F.; Writing-Manuscript Preparation and intellectual input: T.V.A., L.P., D.Y.F.,
661 M.B.K., G.R.D., C.G.S., D.C.H., D.G.J., M.K.P.L., C.L.H.C., and L.H.K.L.;
662 Supervision & Administration: T.V.A., D.Y.F., M.K.P.L., and C.L.H.C.

663

664 **Conflicts Of Interest**

665 The authors declare that the research was conducted in the absence of any
666 commercial or financial relationships that could be construed as a potential conflict of
667 interest.

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876 **FIGURE LEGENDS**

877 **Figure 1: Effect of chronic cerebral hypoperfusion on inflammasome activation**
878 **and cell death in the cerebral cortex and hippocampus over time following**
879 **BCAS.** (a & b), representative immunoblots and quantification illustrating increased
880 levels of activated inflammasome effector proteins such as cleaved caspase-1 (p20),
881 -8, and -11, and maturation of downstream effector targets, IL-1 β and IL-18, in the
882 cerebral cortex. (c & d), representative immunoblots and quantification illustrating
883 increased levels of activated inflammasome effector proteins such as cleaved
884 caspase-1 (p20, p33), -8, and -11, and maturation of downstream effector targets, IL-
885 1 β and IL-18, in the hippocampus. (e & f), representative immunoblots and
886 quantification illustrating increases in the expression of apoptotic marker cleaved
887 caspase-3 and pyroptotic marker N-terminal GSDMD in the cerebral cortex. (g & h),
888 representative immunoblots and quantification illustrating increases in the expression
889 of apoptotic marker cleaved caspase-3 and pyroptotic marker N-terminal GSDMD in
890 the hippocampus. β -actin was used as a loading control. Data are represented as
891 mean \pm S.E.M. n=6-7 mice in each experimental group. *P<0.05 compared with
892 Sham; **P<0.01 compared with Sham; ***P<0.001 compared with Sham.
893 Abbreviations: BCAS, bilateral common carotid artery stenosis; Cl, cleaved; FL, full
894 length; NT, N-terminal; GSDMD, gasdermin D; GSDME, gasdermin E.

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896

897 **Figure 2: Effect of chronic cerebral hypoperfusion on the levels of glial**
898 **activation, white matter integrity and hippocampal neuronal density in the**
899 **cerebral cortex and hippocampus following BCAS.** (a), representative
900 immunoblots and quantification illustrating increased microglial activation due to
901 increased levels of Iba-1 in the cerebral cortex over time following BCAS. Data are
902 represented as mean \pm S.E.M. n=6-7 mice in each experimental group. β -actin was
903 used as a loading control. *P<0.05 compared with Sham. (b), representative
904 immunofluorescence analysis of Iba-1 and GFAP in the cerebral cortex provide
905 supporting evidence of microglial activation following BCAS. Magnification x 100.
906 Scale bar, 20 μ m. Images were taken under identical exposures and conditions. (c),
907 representative immunoblots and quantification illustrating increased astroglia
908 activation due to increased levels of GFAP in the hippocampus over time following
909 BCAS. Data are represented as mean \pm S.E.M. n=6-7 mice in each experimental

910 group. β -actin was used as a loading control. * $P < 0.05$ compared with Sham;
911 ** $P < 0.01$ compared with Sham; *** $P < 0.001$ compared with Sham. (d), representative
912 immunofluorescence analysis of Iba-1 and GFAP in the hippocampus provide
913 supporting evidence of astroglia activation following BCAS. Magnification x 100.
914 Scale bar, 20 μ m. Images were taken under identical exposures and conditions. (e &
915 f), representative merged immunofluorescence images of DAPI, CD86 and CD206
916 co-localized within microglia (Iba-1 positive) in the cerebral cortex and hippocampus
917 of WT controls following BCAS. Representative merged immunofluorescence images
918 illustrate the activation of CD86 (M1) and CD206 (M2) positive microglia in the
919 cerebral cortex (e) and hippocampus (f) following BCAS. (g & h), representative
920 merged immunofluorescence images of DAPI, Complement C3 (C3) and S100A10
921 within astrocytes (GFAP positive) in the cerebral cortex and hippocampus of WT
922 controls following BCAS. Other than colocalization of S100A10 (A2) within astrocytes
923 in cerebral cortex (g), no substantial co-localization of C3 (A1) was observed in the
924 cerebral cortex and hippocampus following BCAS. This illustrates activation of A2
925 astrocytes in the cerebral cortex (g) upon BCAS. Zoom Magnification x 100. Scale
926 bar, 20 μ m. (i & j), representative immunofluorescence images illustrating a loss of
927 myelin due to decreased levels of MBP immunoreactivity in the cerebral cortex and
928 hippocampus following BCAS. (k & l), representative immunofluorescence images
929 illustrating a loss of neurons due to decreased levels of MAP2 immunoreactivity in
930 the cerebral cortex and hippocampus following BCAS. Magnification x 20. Scale bar,
931 120 μ m. (m & n), representative Luxol fast blue stained images and quantification
932 illustrating disruption of white matter integrity due to increased myelin rarefaction and
933 white matter lesion formation in the corpus callosum (paramedian), corpus callosum
934 (medial), caudoputamen, internal capsule and optic tract over time following BCAS.
935 The severity of white matter disruption was graded accordingly: Grade 0 = no
936 disruption; Grade 1 = disarrangement of nerve fibers; Grade 2 = formation of marked
937 vacuoles; Grade 3 = disappearance of myelinated fibers. Images were taken under
938 identical exposures and conditions. (o-r), representative crystal violet images and
939 quantification illustrating loss of Nissl positively stained neurons in hippocampal CA1,
940 CA2 and CA3 regions over time following BCAS. Data are represented as mean \pm
941 S.E.M. n=5-7 mice in each experimental group. * $P < 0.05$ compared with Sham;
942 ** $P < 0.01$ compared with Sham; *** $P < 0.001$ compared with Sham. Magnification x 60.
943 Scale bar, 20 μ m. Images were taken under identical exposures and conditions.

944 Abbreviations: BCAS, bilateral common carotid artery stenosis; CD86, cluster of
945 differentiation 86; CD206, cluster of differentiation 206; C3, complement component
946 3; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adaptor
947 molecule-1; MBP, myelin basic protein; MAP2, microtubule-associated protein 2;
948 S100A10, S100 calcium-binding protein A10.

949

950 **Figure 3: Effect of chronic cerebral hypoperfusion on inflammasome-mediated**
951 **programmed cell death in AIM2 KO mice following BCAS.** (a), quantification
952 illustrating increased serum concentrations of the AIM2 receptor ligand, cell free
953 double stranded DNA (dsDNA), over time in wild-type mice following BCAS. Data are
954 represented as mean \pm S.E.M. n=5-7 mice in each experimental group. *P<0.05
955 compared with Sham. (b-e), representative immunoblots and quantification
956 illustrating suppression of inflammasome activation due to decreased expression of
957 cleaved caspase-1 (p20) at Day 15 and 30, and maturation of IL-1 β cytokine
958 production at Day 30 in the cerebral cortex (b & c) and hippocampus (d & e) of AIM2
959 KO mice compared to WT controls following BCAS. (f-i), representative immunoblots
960 and quantification illustrating attenuation of apoptotic and pyroptotic cell death due to
961 decreased expression of cleaved caspase-3 and GSDMD-NT, respectively, at Day
962 30 in the cerebral cortex (f & g) and hippocampus (h & i) of AIM2 KO mice compared
963 to WT controls following BCAS. β -actin was used as a loading control. Data are
964 represented as mean \pm S.E.M. n=5-8 mice in each experimental group. *P<0.05
965 compared with WT Sham; **P<0.01 compared with WT Sham; ***P<0.001 compared
966 with WT Sham. ⁺P<0.05 compared with WT BCAS; ⁺⁺P<0.01 compared with WT
967 BCAS; ⁺⁺⁺P<0.001 compared with WT BCAS. (j & k), representative merged
968 immunofluorescence images of DAPI, cleaved caspase-1 p10 (CC1), cleaved
969 caspase-3 (CC3) and GSDMD co-localized within neurons (MAP2 positive), microglia
970 (Iba-1 positive), oligodendrocytes (OLIG2 positive) and endothelial cells (PECAM-1
971 positive) in the cerebral cortex and hippocampus of WT controls following BCAS. No
972 substantial co-localization of cleaved caspase-1 p10, cleaved caspase-3 and
973 GSDMD was observed in astrocytes (GFAP positive) in the cerebral cortex and
974 hippocampus of WT controls following BCAS. (j & k), representative merged
975 immunofluorescence images illustrate a reduction in inflammasome activation, and
976 apoptotic and pyroptotic cell death due to decreased expression levels of cleaved
977 caspase-1, cleaved caspase-3 and GSDMD, respectively, in both neurons and

978 microglia in the cerebral cortex (j) and hippocampus (k) in AIM2 KO mice compared
979 to WT controls following BCAS. Zoom Magnification x 100. Scale bar, 20 μ m.
980 Images were taken under identical exposures and conditions. Abbreviations: BCAS,
981 bilateral common carotid artery stenosis; Cl, cleaved; FL, full length; NT, N-terminal;
982 WT, wild-type; KO, knock out; CC1, cleaved caspase-1; CC3, cleaved caspase-3;
983 GSDMD, Gasdermin D; MAP2, microtubule-associated protein 2; Iba-1, ionized
984 calcium binding adaptor molecule-1; OLIG2, oligodendrocyte transcription factor 2;
985 GFAP, glial fibrillary acidic protein; PECAM-1, platelet endothelial cell adhesion
986 molecule-1.

987

988 **Figure 4: Effect of chronic cerebral hypoperfusion on glial activation and**
989 **myelin expression in the cerebral cortex and hippocampus of AIM2 KO mice**
990 **following BCAS.** (a & b, d & e), representative immunoblots and quantification
991 illustrating resistance to microglial activation due to decreased expression of Iba-1 in
992 the cerebral cortex and hippocampus of AIM2 KO mice compared to WT controls
993 following BCAS. No effect on astroglia activation was observed as the expression of
994 GFAP in the cerebral cortex and hippocampus remained unchanged in AIM2 KO
995 mice compared to WT controls following BCAS. β -actin was used as a loading control.
996 Data are represented as mean \pm S.E.M. n=6-8 mice in each experimental group.
997 *P<0.05 compared with WT Sham; **P<0.01 compared with WT Sham. +P<0.05
998 compared with WT BCAS; ++P<0.01 compared with WT BCAS; +++P<0.001
999 compared with WT BCAS. (c & f), representative merged immunofluorescence
1000 images of DAPI, CD86 and CD206 co-localized within microglia (Iba-1 positive) in the
1001 cerebral cortex and hippocampus of WT controls following BCAS. Representative
1002 merged immunofluorescence images illustrate a reduction in M1 (CD86 positive) and
1003 M2 (CD206 positive) microglial activation due to decreased expression levels of
1004 CD86 and CD206 respectively, in the cerebral cortex (c) and hippocampus (f) in
1005 AIM2 KO mice compared to WT controls following BCAS. (c & f), representative
1006 merged immunofluorescence images of DAPI, Complement C3 (C3) and S100A10
1007 within astrocytes (GFAP positive) in the cerebral cortex and hippocampus of WT
1008 controls following BCAS. Other than colocalization of S100A10 (A2) within astrocytes
1009 in the cerebral cortex (c), no substantial co-localization of C3 (A1) was observed in
1010 the cerebral cortex (c) and hippocampus (f) of following BCAS. Representative
1011 merged immunofluorescence images illustrate a reduction in A2 (S100A10 positive)

1012 astrocytes activation due to decreased expression levels of S100A10 in the cerebral
1013 cortex (c) in AIM2 KO mice compared to WT controls following BCAS. (g & h, j & k),
1014 representative immunoblots and quantification illustrating resistance to myelin loss
1015 due to increased expression of MBP in the cerebral cortex (g) and hippocampus (j) in
1016 AIM2 KO mice compared to WT controls following BCAS. β -actin was used as a
1017 loading control. Data are represented as mean \pm S.E.M. n=5-7 mice in each
1018 experimental group. *P<0.05 compared with WT Sham; +P<0.05 compared with WT
1019 BCAS; ++P<0.01 compared with WT BCAS (i & l), representative
1020 immunofluorescence images illustrating resistance to myelin loss due to increased
1021 MBP immunoreactivity in the cerebral cortex (i), and hippocampal CA1, CA2 and
1022 CA3 regions (l) in AIM2 KO mice compared to WT controls following BCAS.
1023 Magnification x 100. Scale bar, 20 μ m. Images were taken under identical exposures
1024 and conditions. Abbreviations: BCAS, bilateral common carotid artery stenosis; CD86,
1025 cluster of differentiation 86; CD206, cluster of differentiation 206; C3, complement
1026 component 3; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding
1027 adaptor molecule-1; KO, knock out; S100A10, S100 calcium-binding protein A10.

1028

1029 **Figure 5: Effect of chronic cerebral hypoperfusion on cortical white matter**
1030 **integrity and hippocampal neuronal density in AIM2 KO mice following BCAS.**

1031 (a-e), representative Luxol fast blue stained images and quantification illustrating
1032 preserved white matter integrity due to decreased myelin rarefaction and white
1033 matter lesion formation in the corpus callosum (paramedian), corpus callosum
1034 (medial), caudoputamen and internal capsule in AIM2 KO mice compared with WT
1035 controls following BCAS. No significant difference in white matter integrity was
1036 observed in the optic tract between AIM2 KO mice compared with WT controls
1037 following 30 days of BCAS. The severity of white matter disruption was graded
1038 accordingly: Grade 0 = no disruption; Grade 1 = disarrangement of nerve fibres;
1039 Grade 2 = formation of marked vacuoles; Grade 3 = disappearance of myelinated
1040 fibres. (f-h), representative crystal violet images and quantification illustrating
1041 increased Nissl positively stained neurons in hippocampal CA1, CA2 and CA3
1042 regions in AIM2 KO mice compared to WT controls following BCAS. Data are
1043 represented as mean \pm S.E.M. n=5-7 mice in each experimental group. *P<0.05
1044 compared with WT Sham; **P<0.01 compared with WT Sham; ***P<0.001 compared
1045 with WT Sham. +P<0.05 compared with WT BCAS; ++P<0.01 compared with WT

1046 BCAS; $^{+++}P < 0.001$ compared with WT BCAS. Magnification x 60. Scale bar, $20 \mu\text{m}$.
1047 Images were taken under identical exposures and conditions. Abbreviations: BCAS,
1048 bilateral common carotid artery stenosis; WT, wild type; KO, knock out; WML, white
1049 matter lesion.

1050

1051

1052 **Figure 6: Effect of chronic cerebral hypoperfusion on explorative locomotive**
1053 **behavior and spatial memory in AIM2 KO mice following BCAS.** (a-d),

1054 quantifications illustrating an open field test conducted on all mice to examine the
1055 locomotor ability and level of anxiety by measuring the total distance travelled (mm)
1056 and average speed of movement (mm/s); total time (sec) and distance travelled (mm)

1057 in the outer and inner zone of the open field. It was shown that WT BCAS mice did
1058 not have a shorter distance travelled (mm) (a) and a lower average speed (mm/s) of
1059 movement (b) when compared to WT Sham controls, indicating no impairment of
1060 locomotor ability. Similarly, there are also no significant difference in the time spent

1061 and distance travelled in the outer and inner zone among WT mice, showing no
1062 BCAS-induced anxiety effect (c & d). However, it was observed that in both sham
1063 and BCAS AIM2 KO mice more time was spent at the outer zone when compared to
1064 WT controls (c), indicating that AIM2 KO mice generally exhibit higher levels of

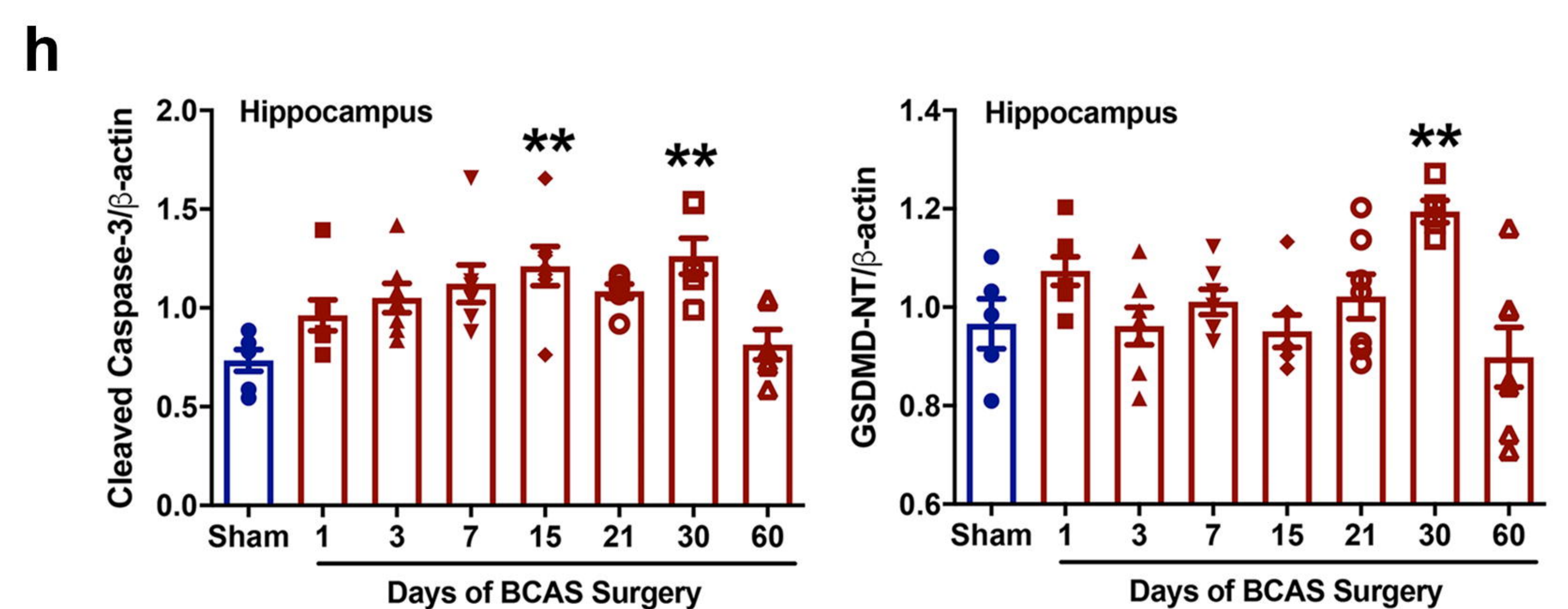
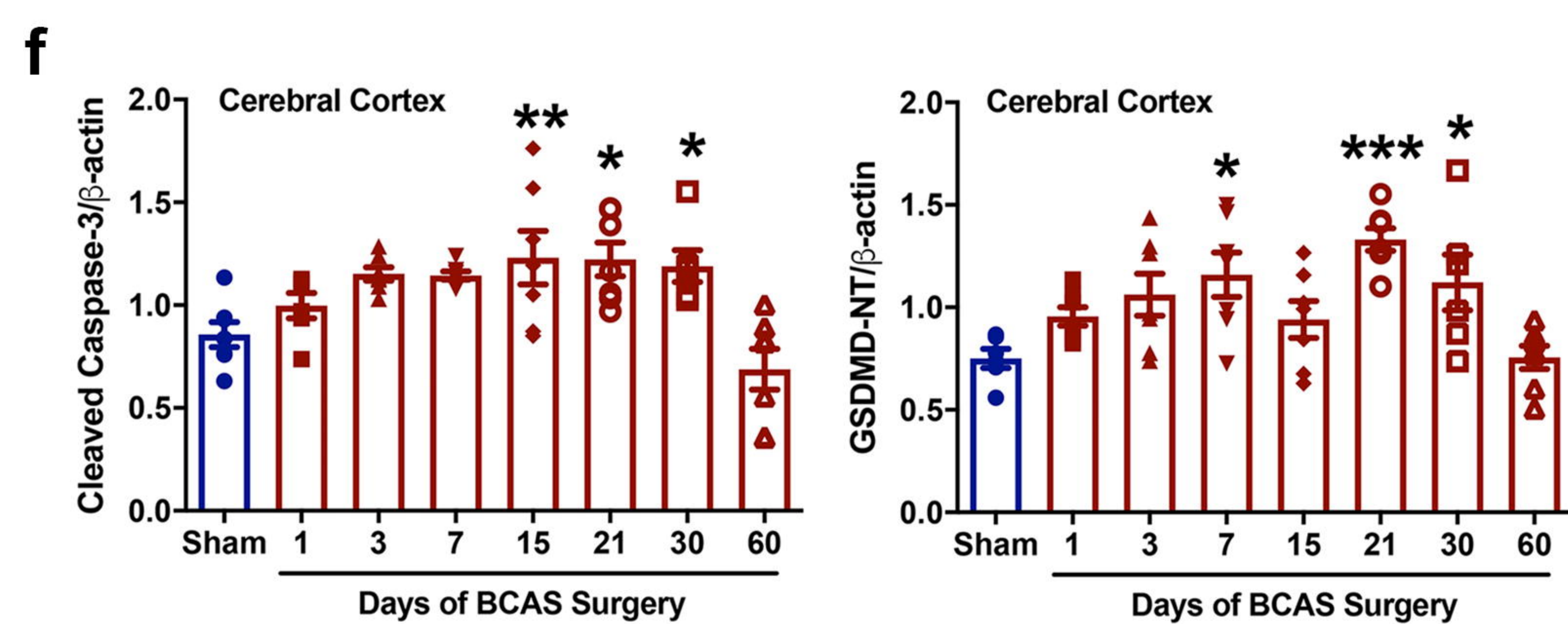
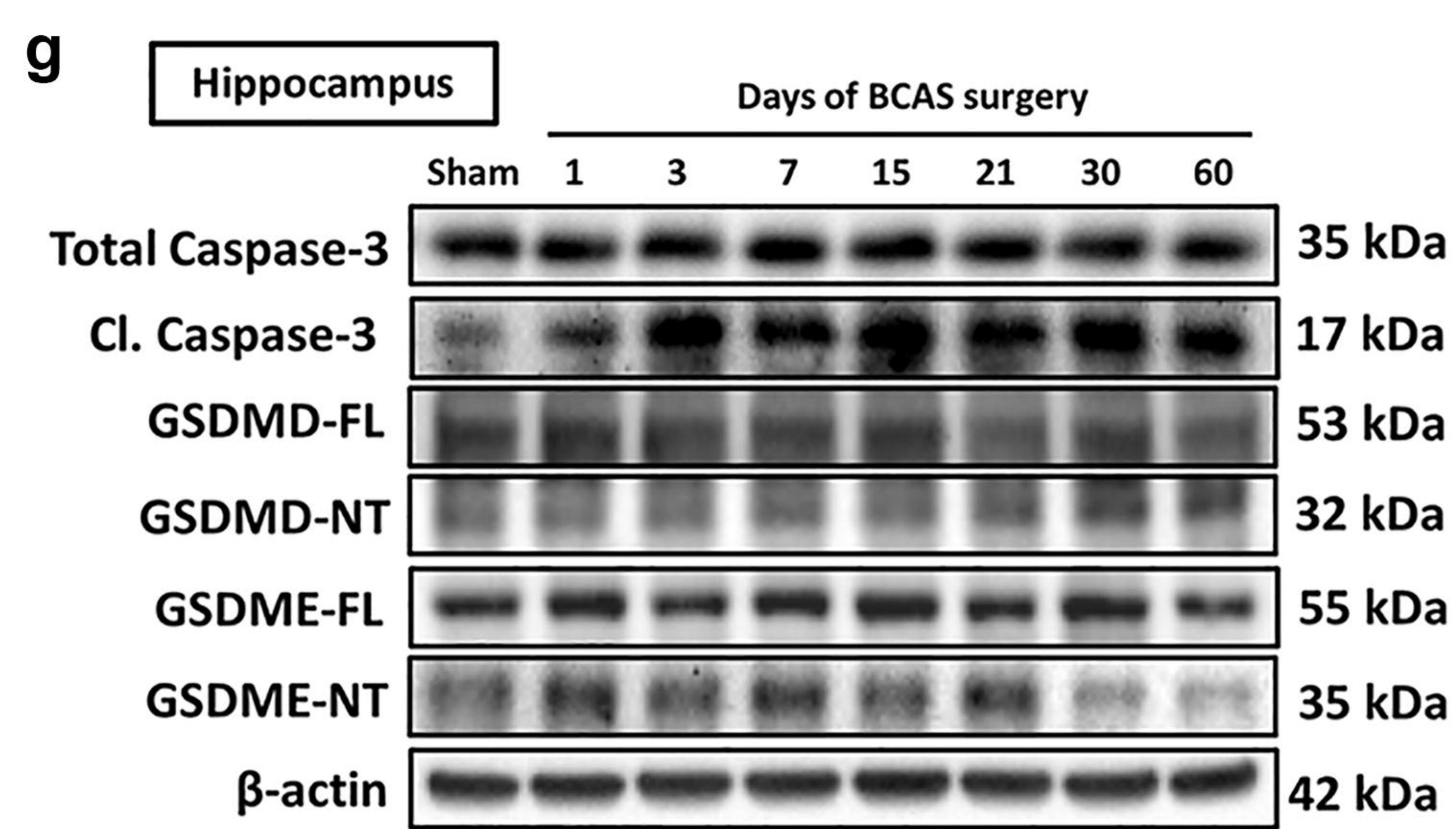
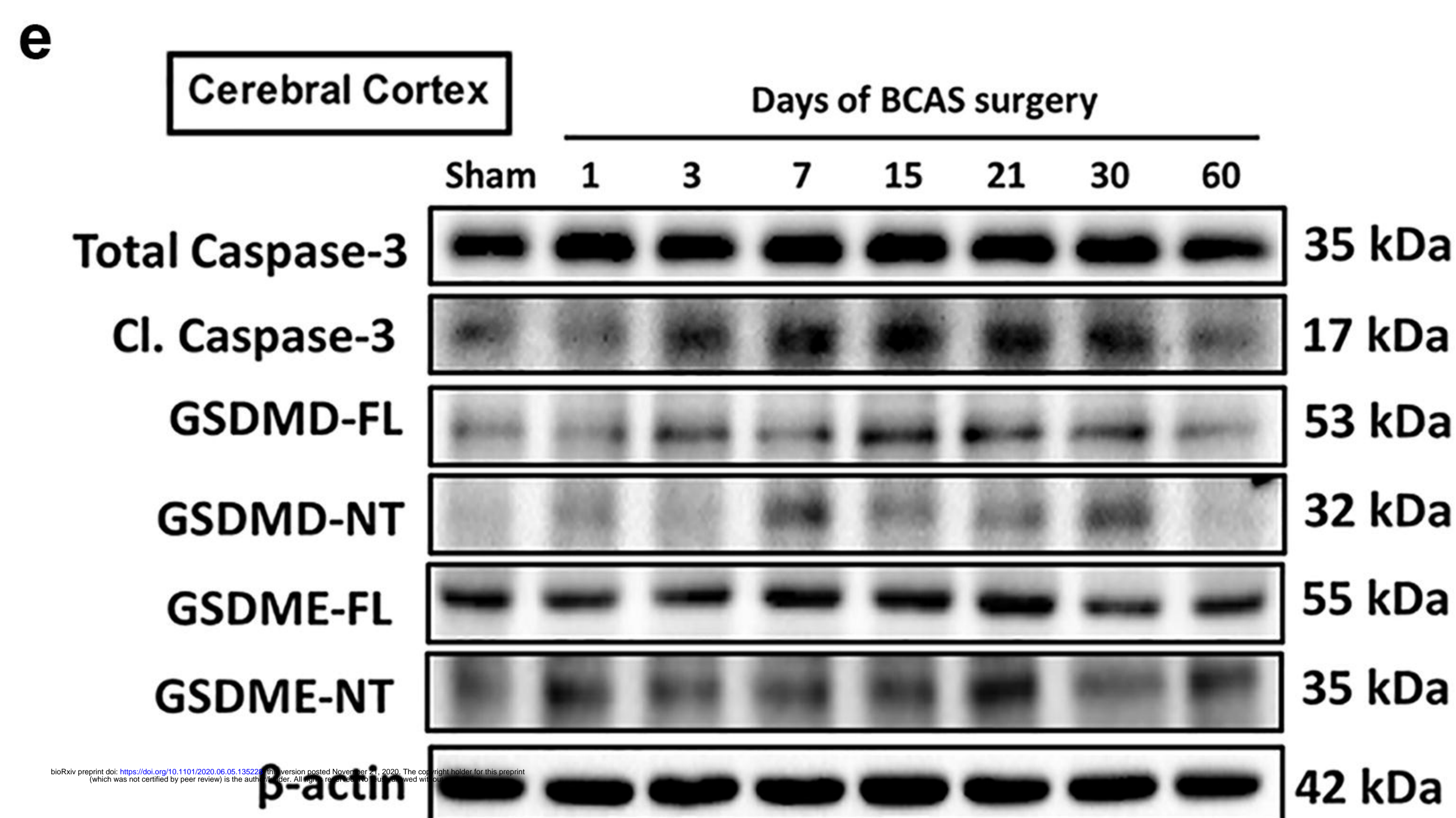
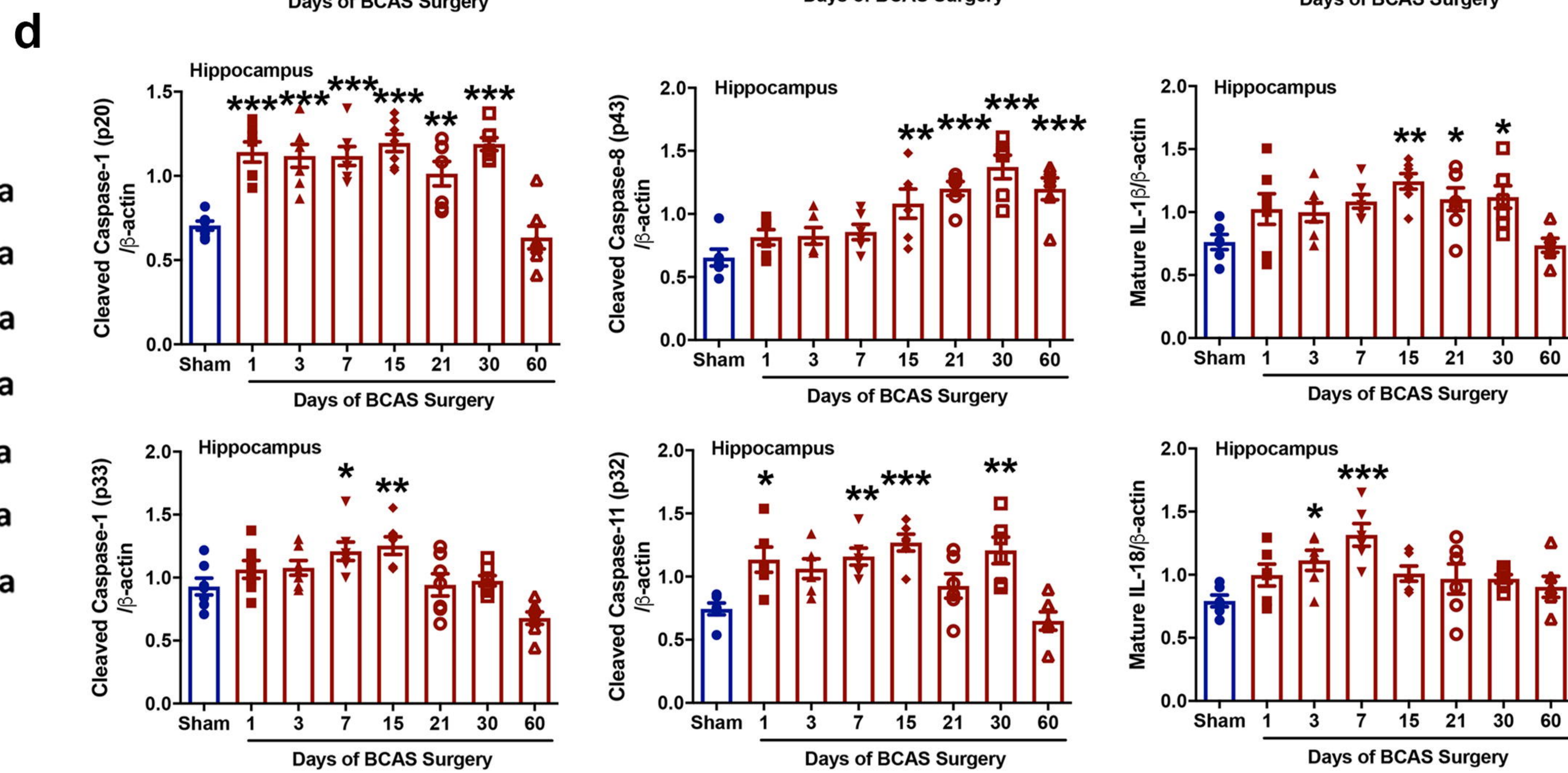
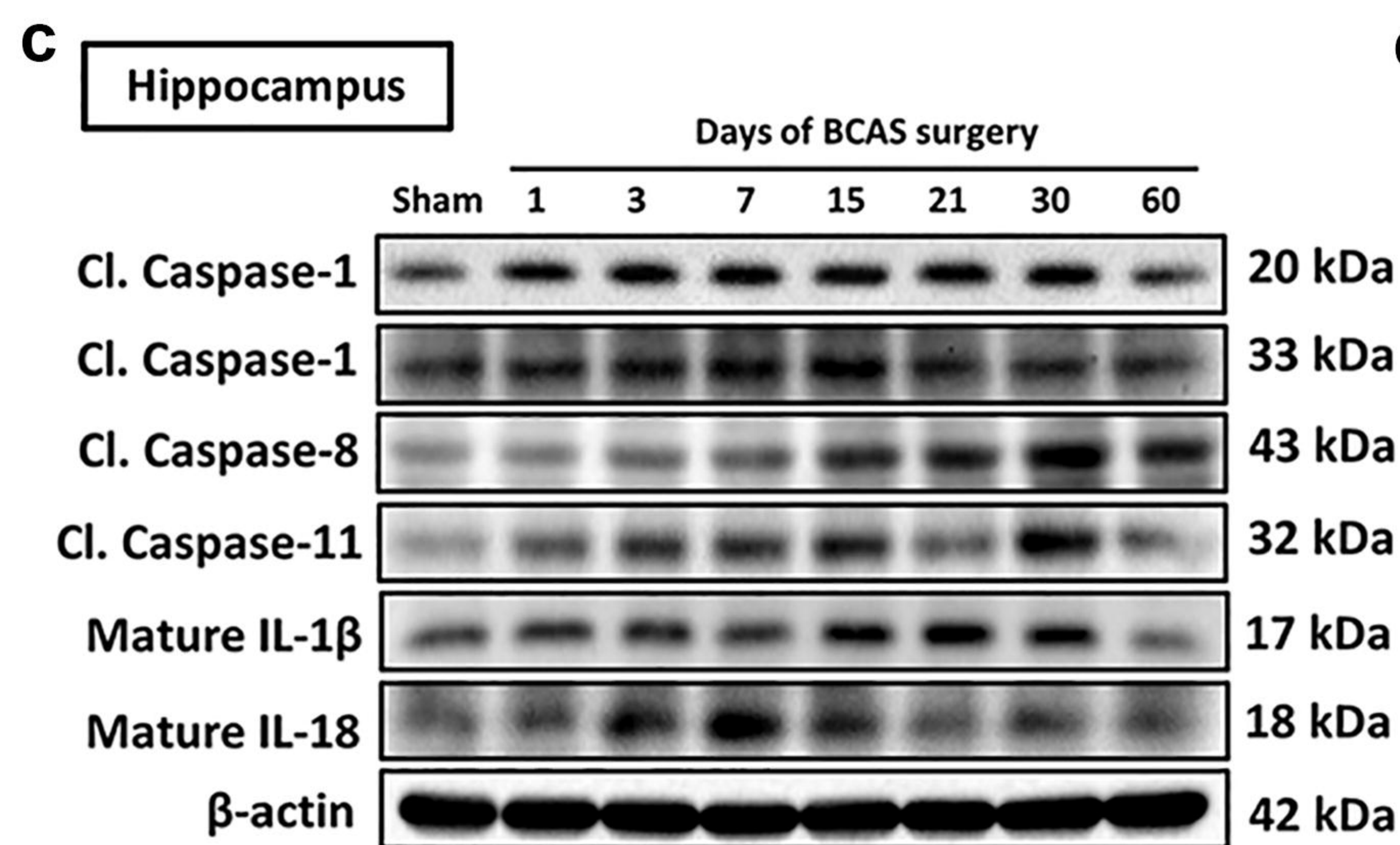
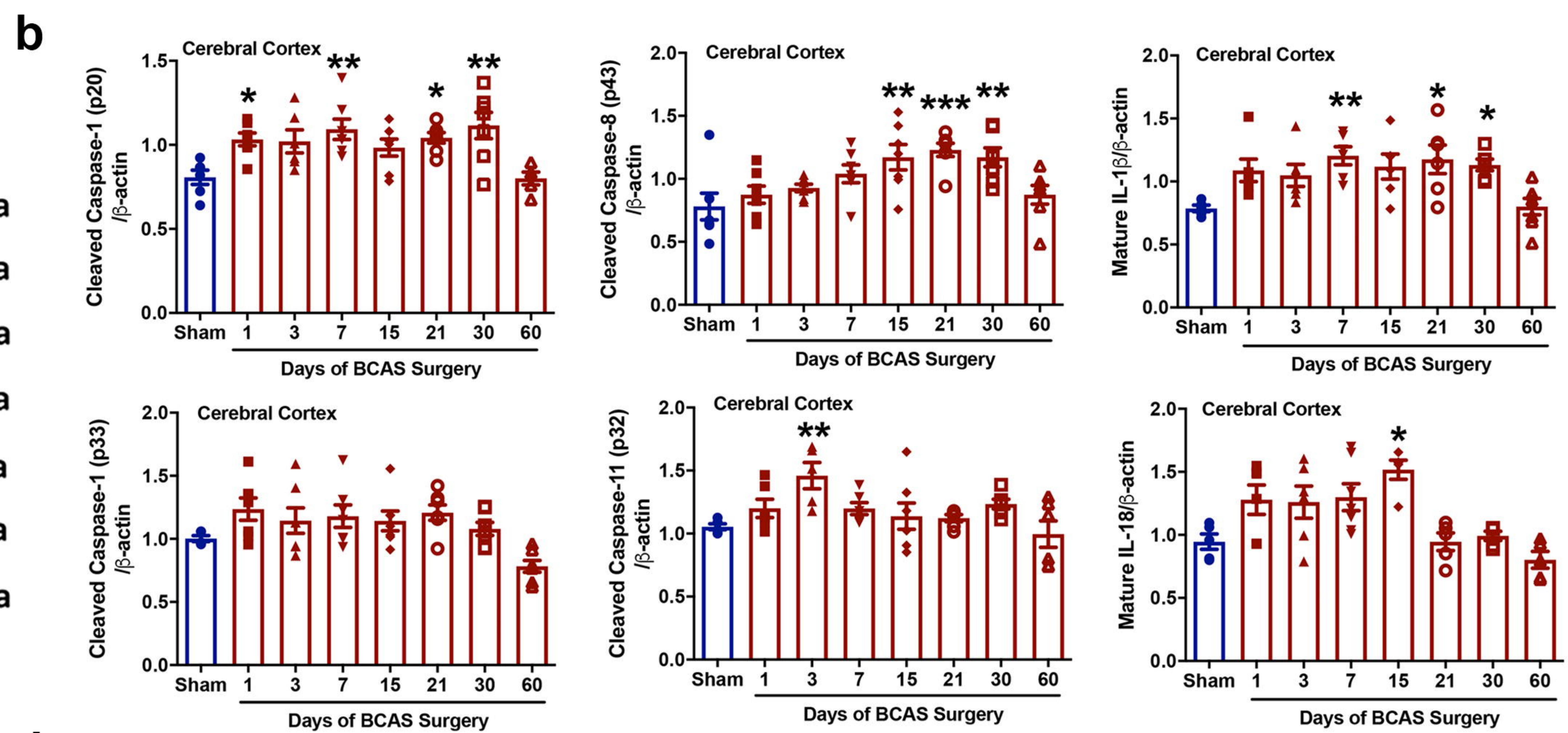
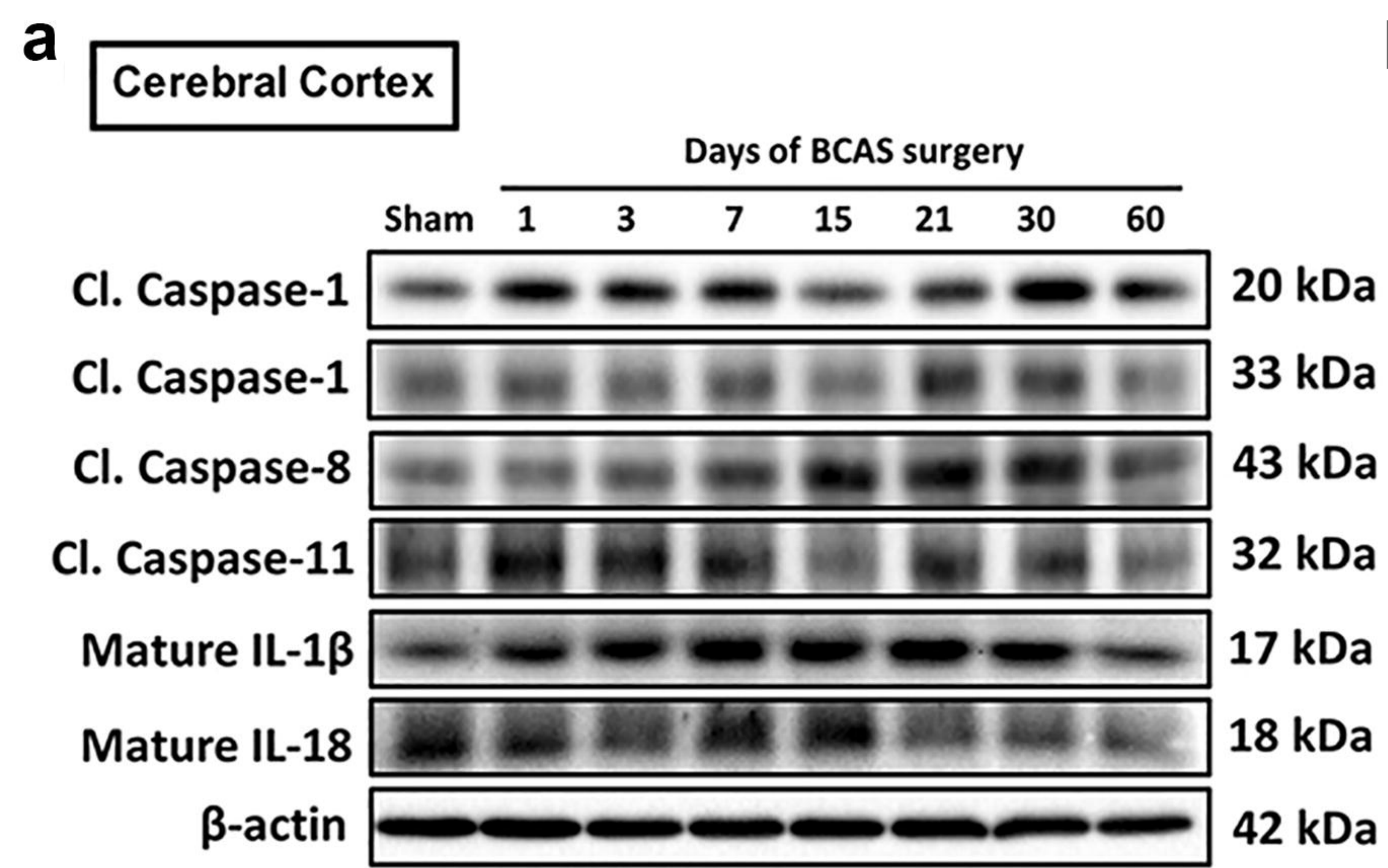
1065 anxiety independent of BCAS. (e-h), quantifications illustrating a Morris water maze
1066 test conducted on all mice to examine spatial learning and memory by measuring the
1067 escape latency (sec), time spent around the platform on probe day (sec), number of
1068 visits in the target quadrant and time spent in target quadrant (sec). (e), in general, a

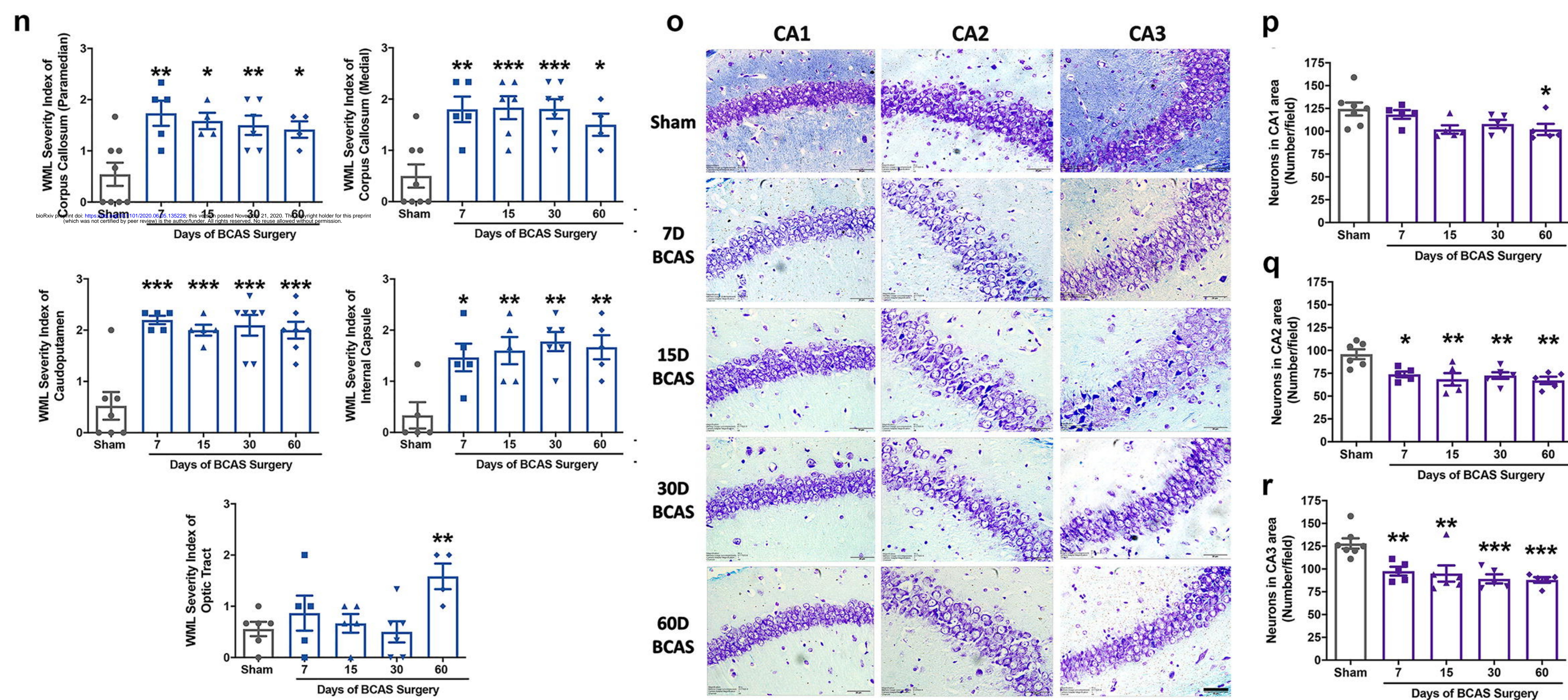
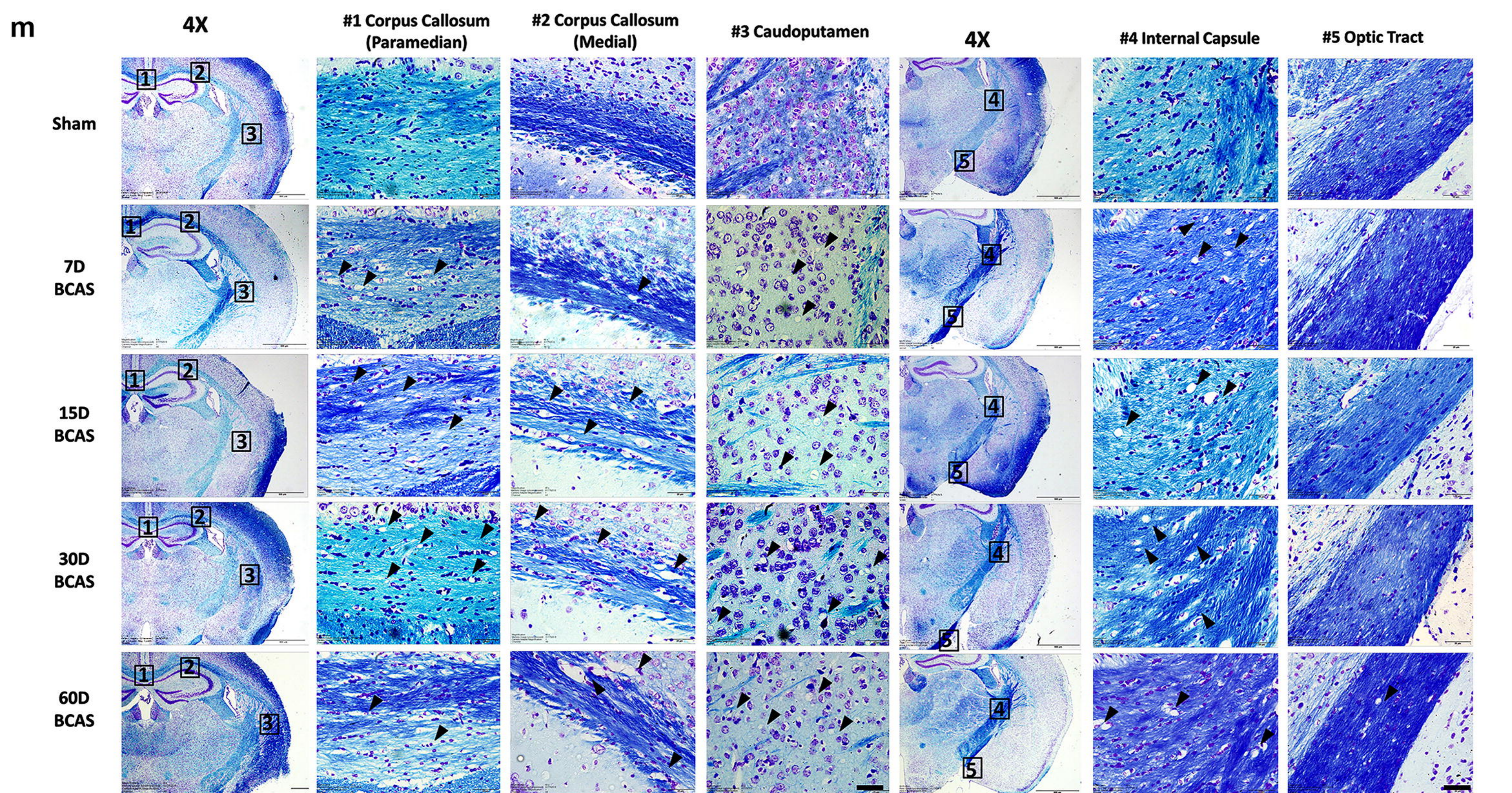
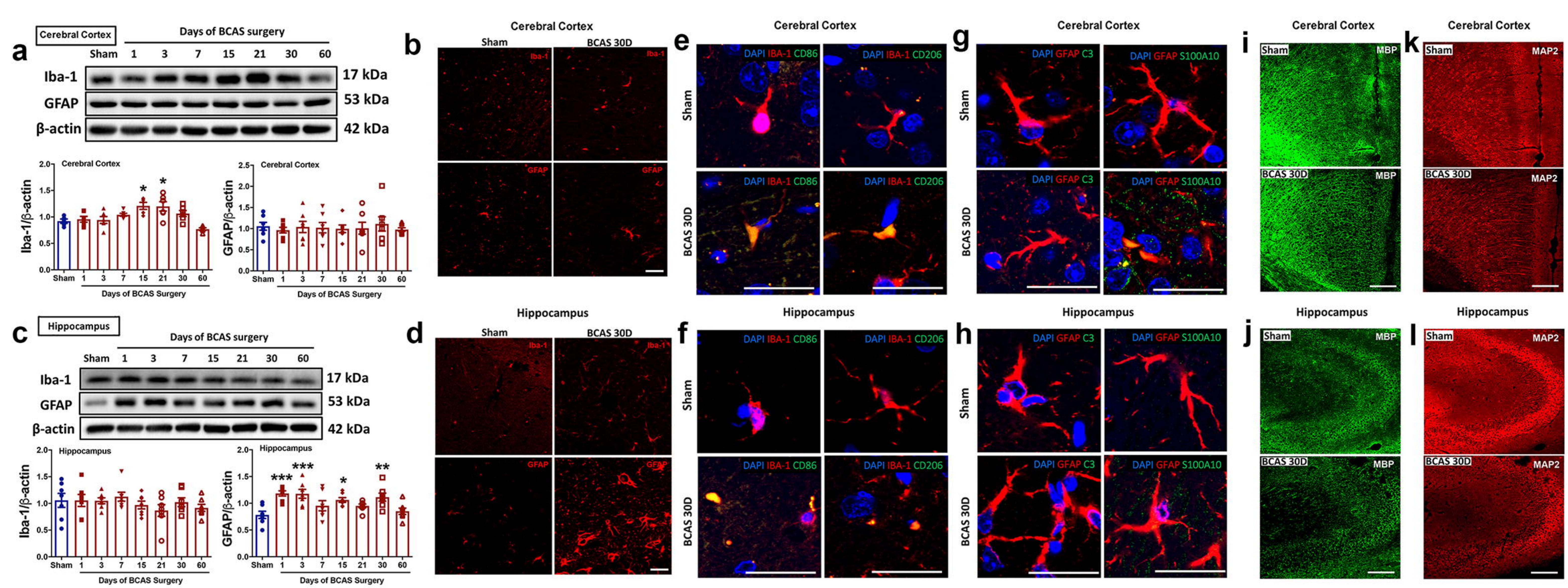
1069 reduction in escape latency (sec) was observed in all mice across the training period.
1070 Specifically, WT Sham, AIM2 KO Sham and AIM2 KO BCAS groups displayed a
1071 trend with a shorter escape latency and steeper declining gradient, which reflected
1072 an improved learning ability when compared to the WT BCAS group. (f-h), in general,

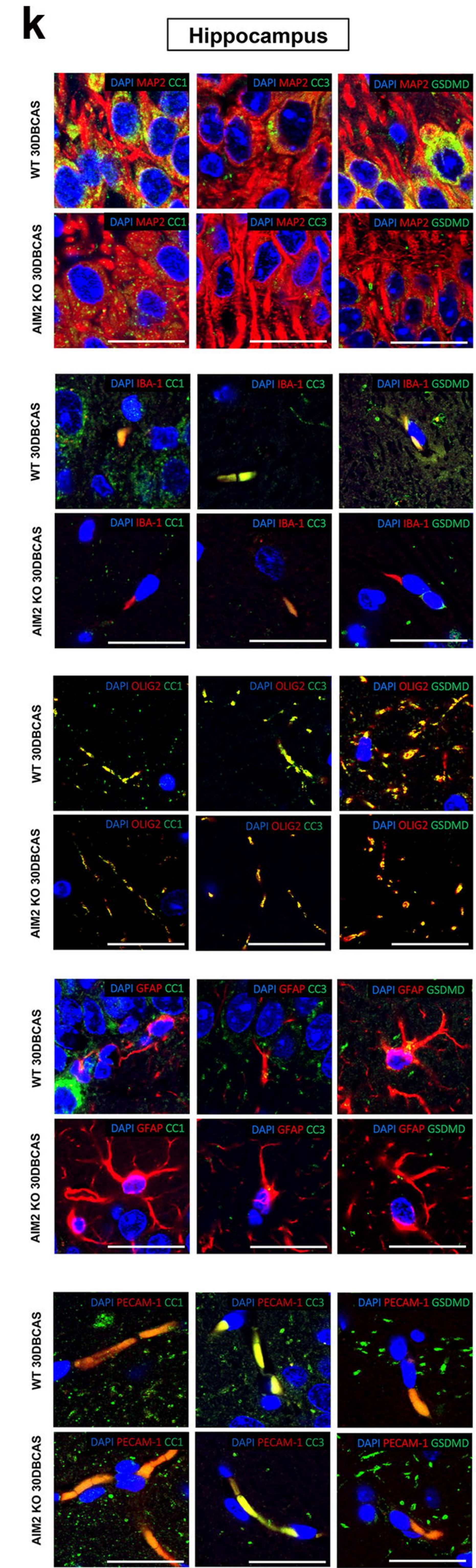
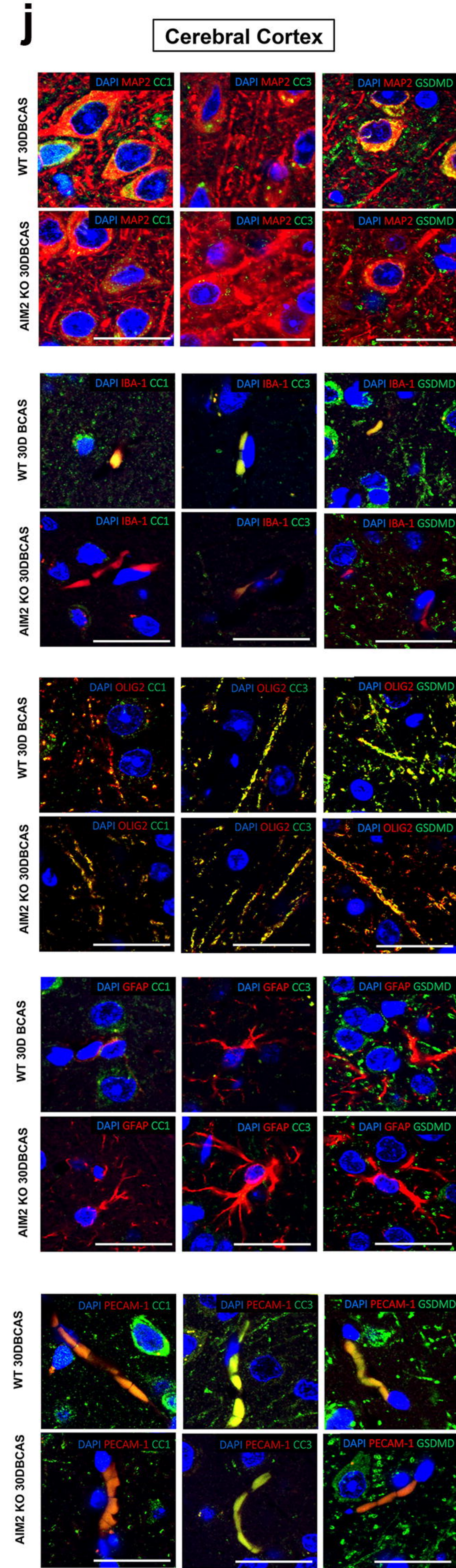
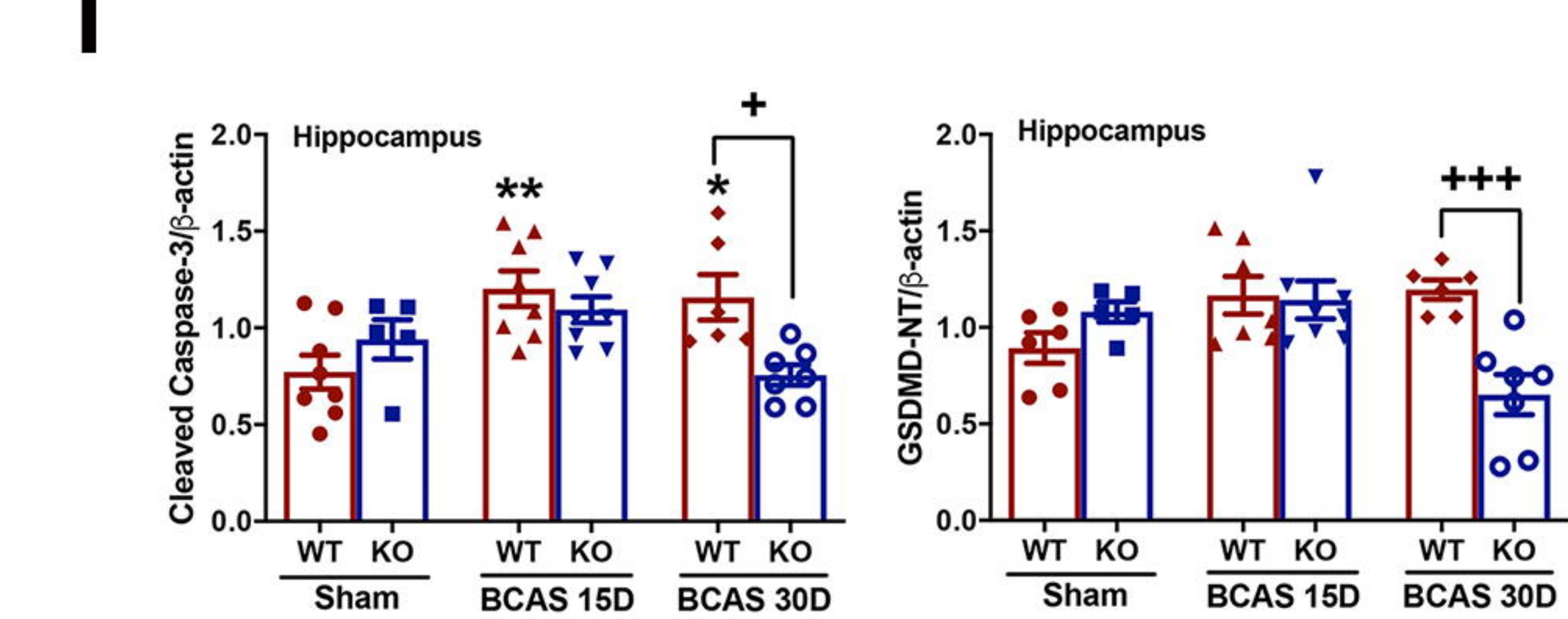
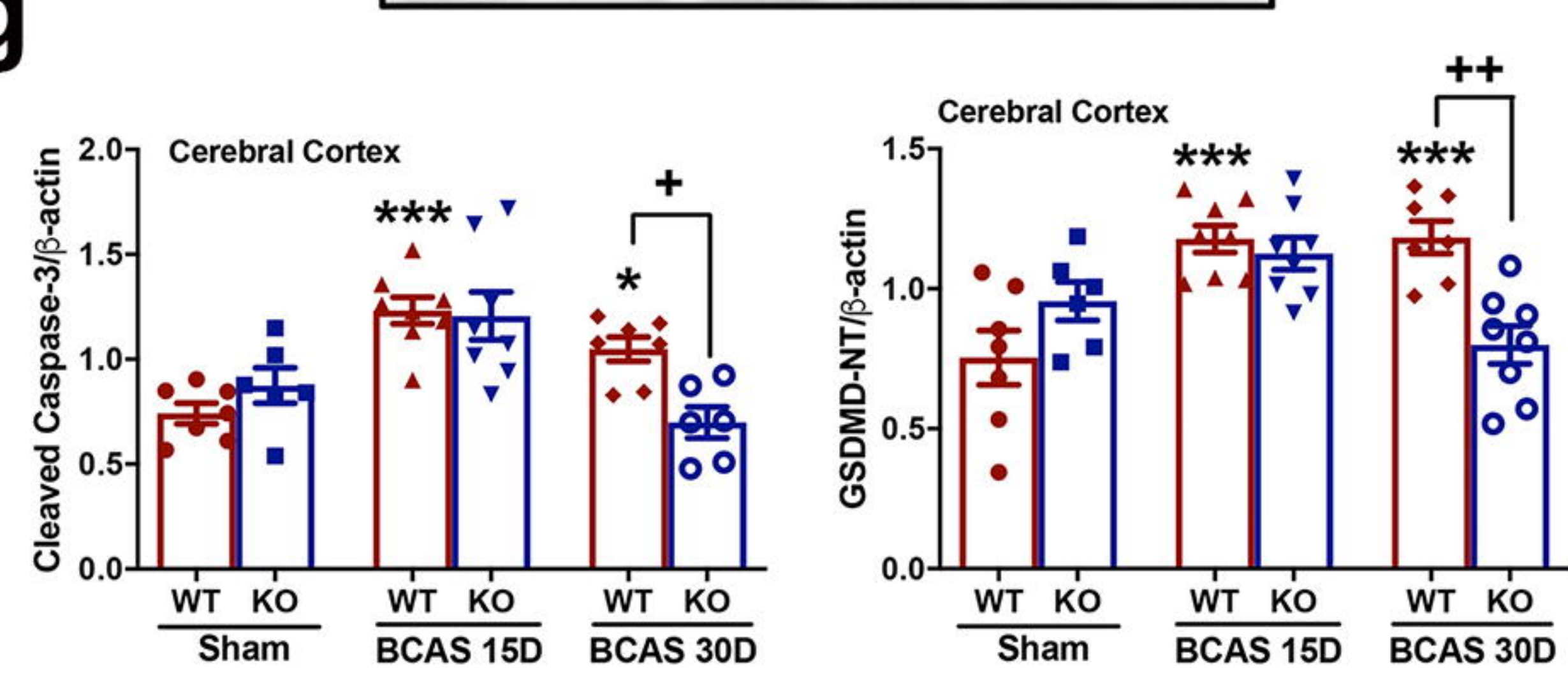
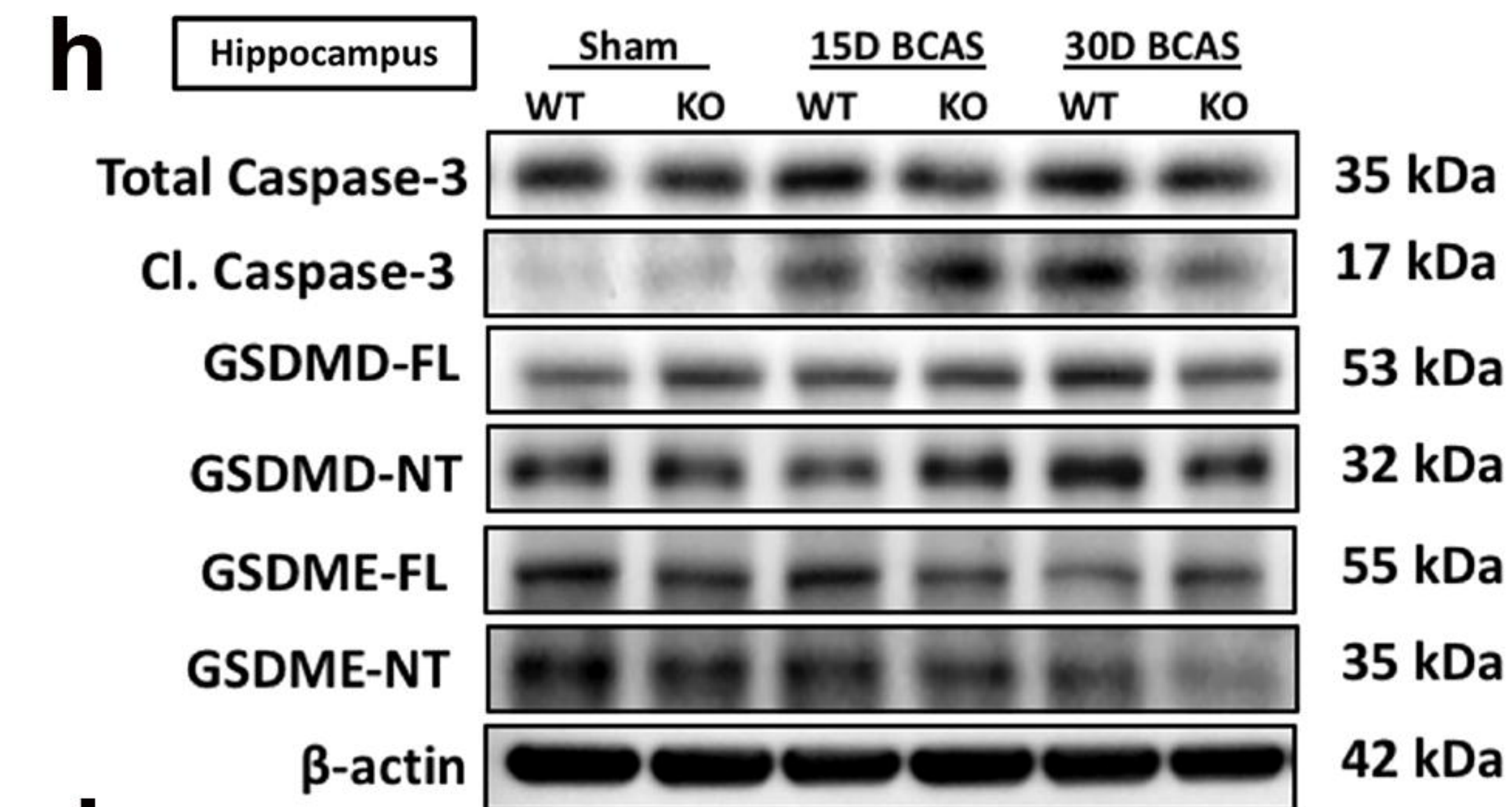
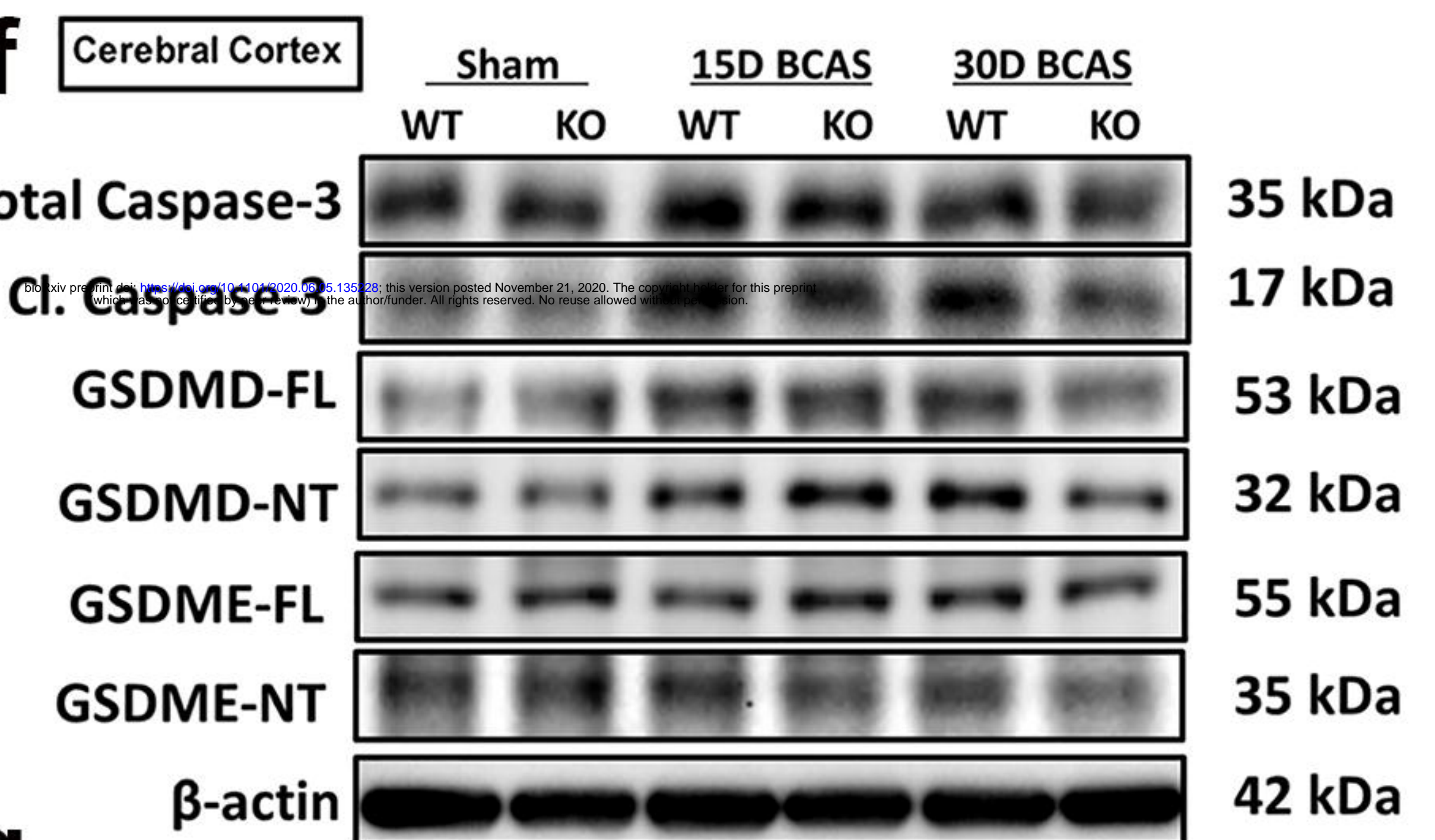
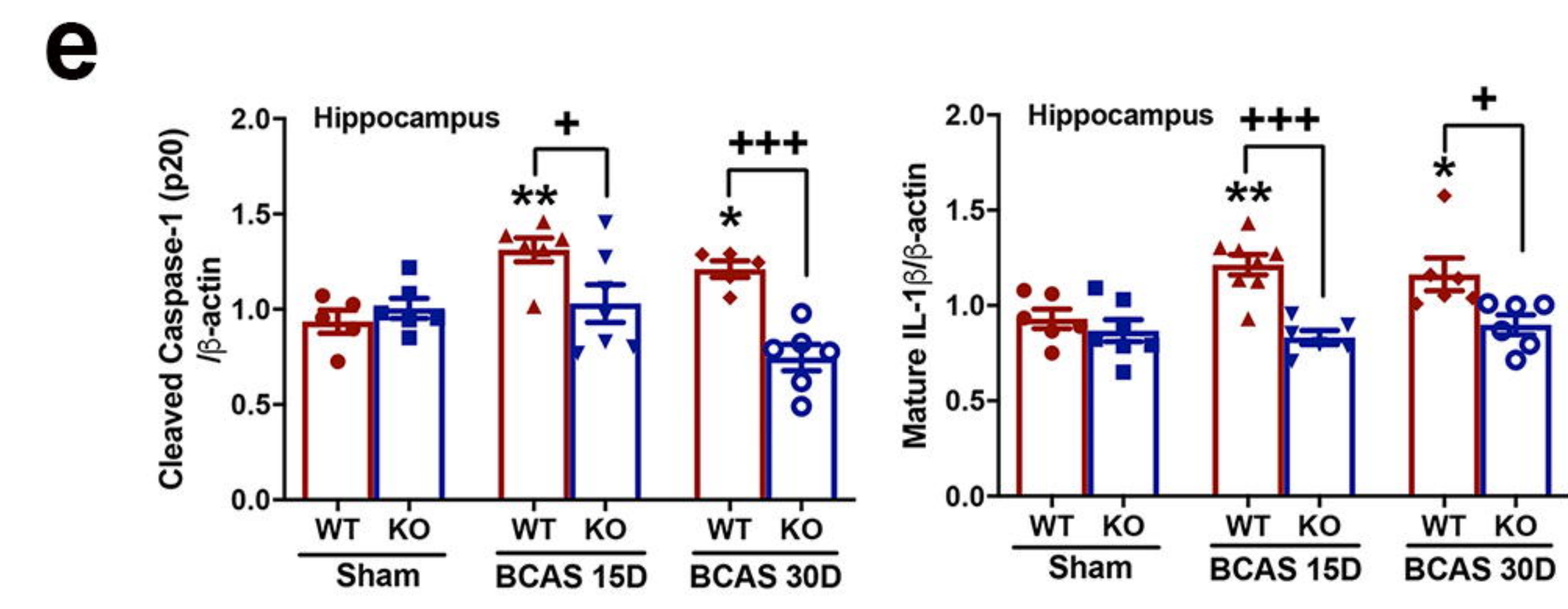
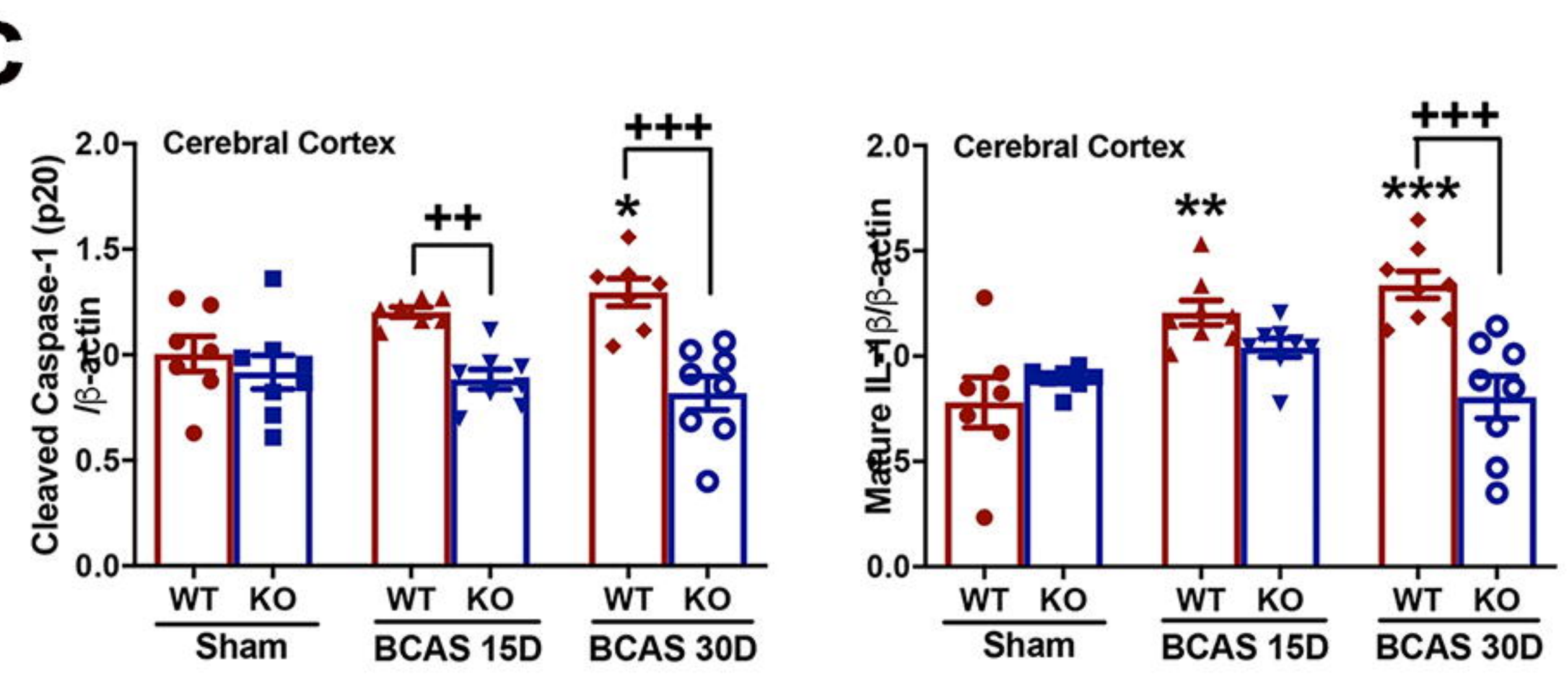
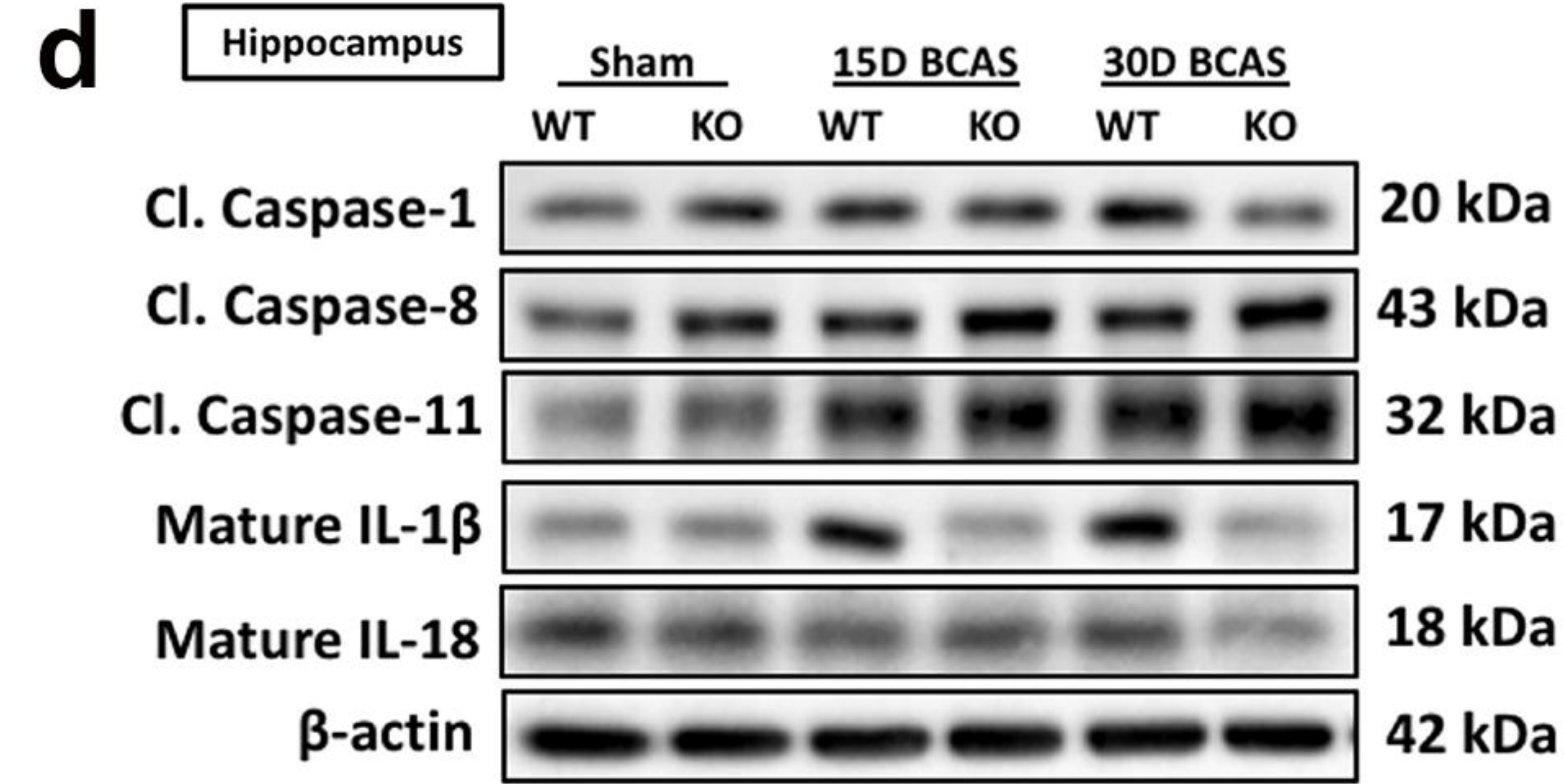
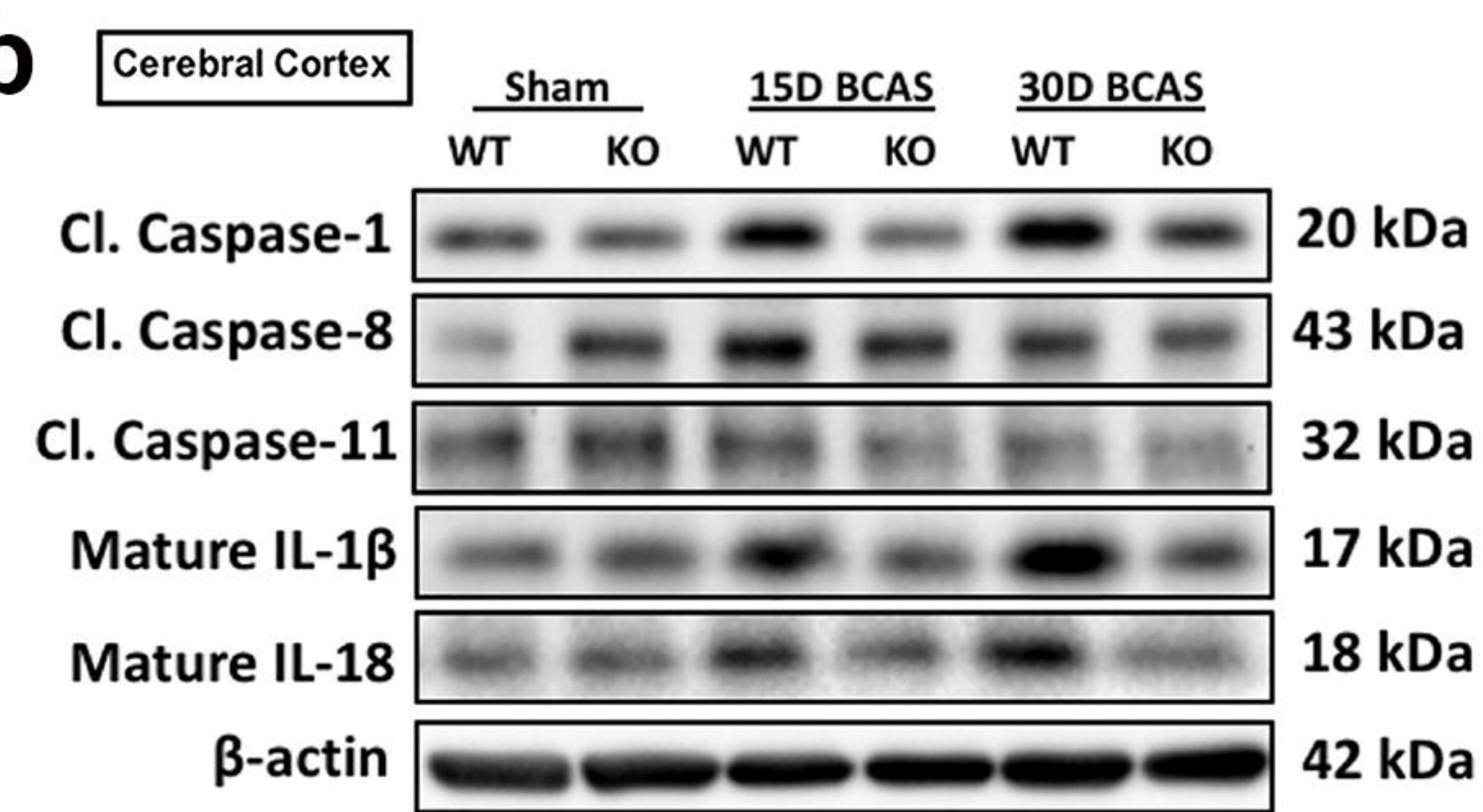
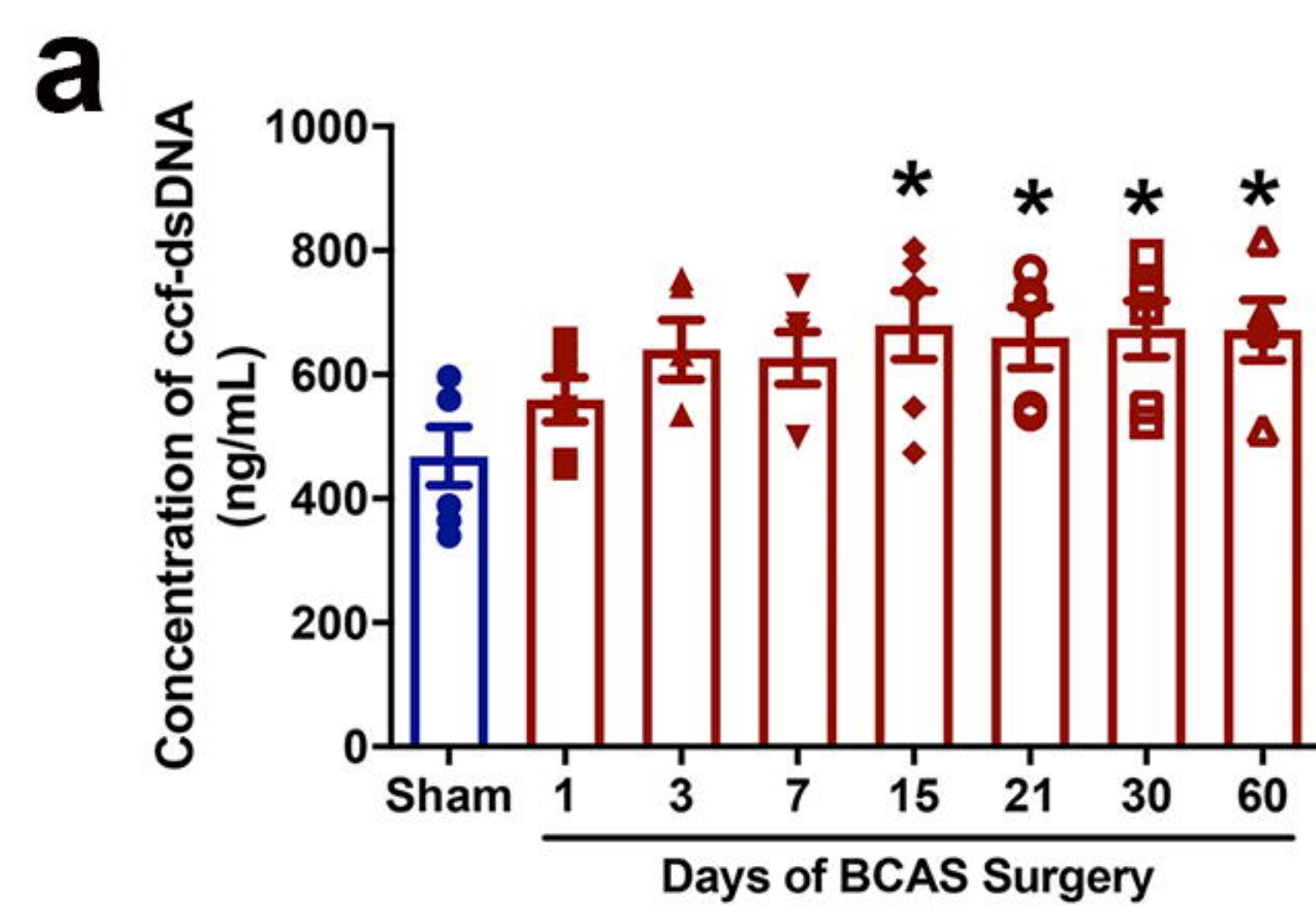
1073 the AIM2 KO BCAS group displayed a trend with a longer time spent around the
1074 platform on probe day and a significantly higher number of visits and time spent in
1075 the target quadrant, which reflected retention of spatial memory when compared to
1076 the WT BCAS group. Data are represented as mean \pm S.E.M. n=9-11 mice in each

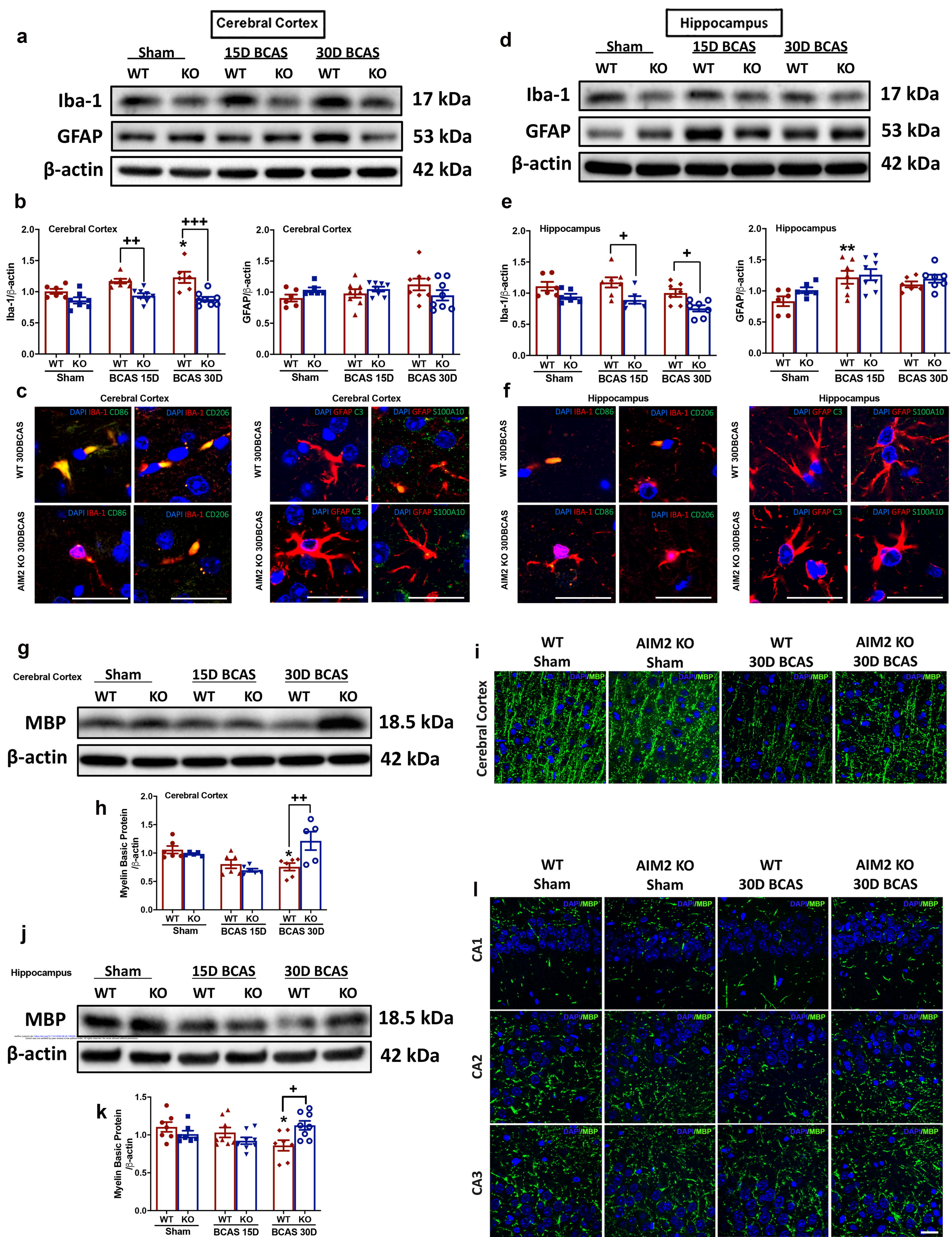
1077 experimental group. * $P < 0.05$ compared with WT Sham. # $P < 0.05$ compared with WT
1078 BCAS; ## $P < 0.01$ compared with WT BCAS; ### $P < 0.001$ compared with WT BCAS.

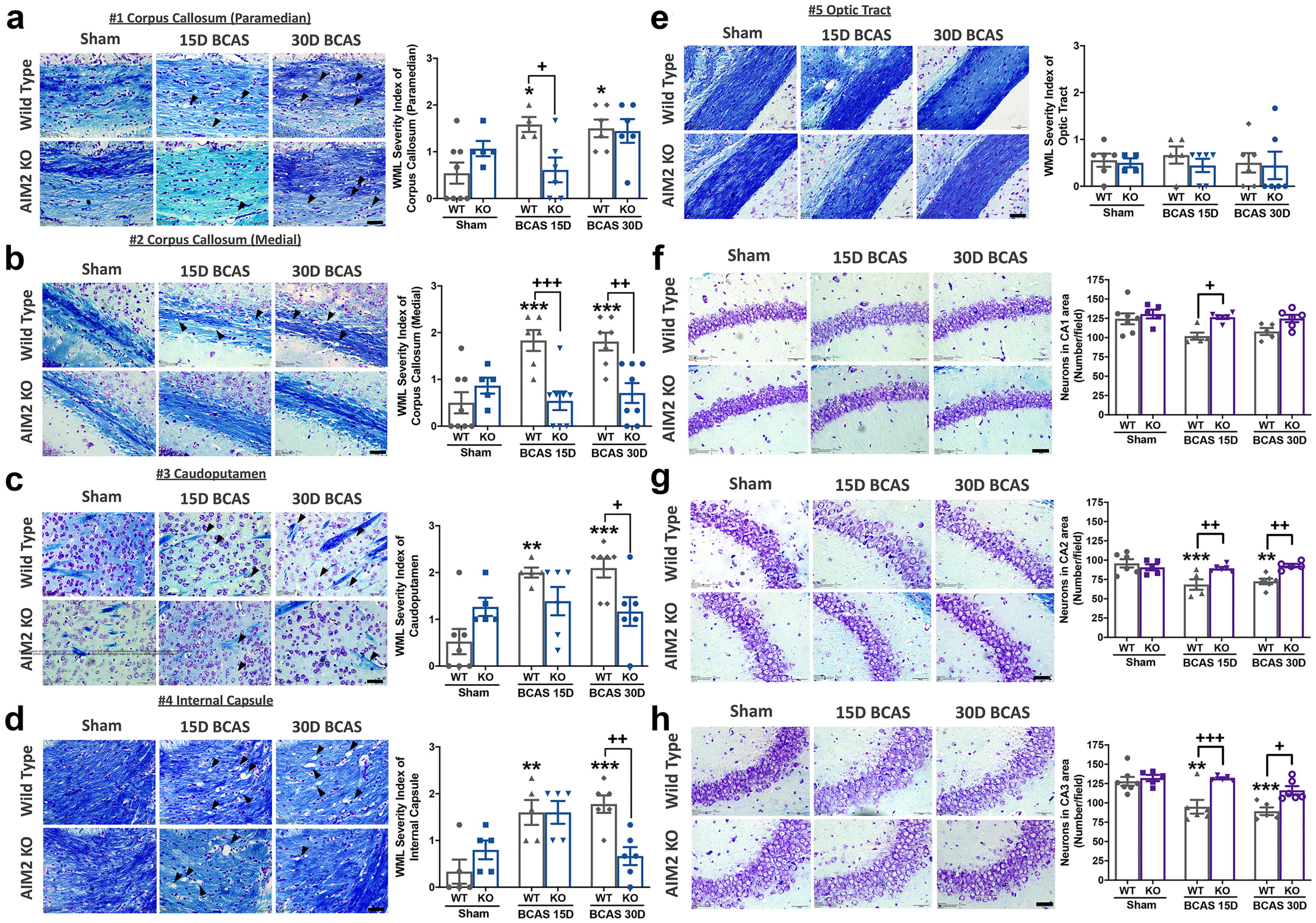
1079 Abbreviations: BCAS, bilateral common carotid artery stenosis; WT, wild type; KO,
1080 knock out.
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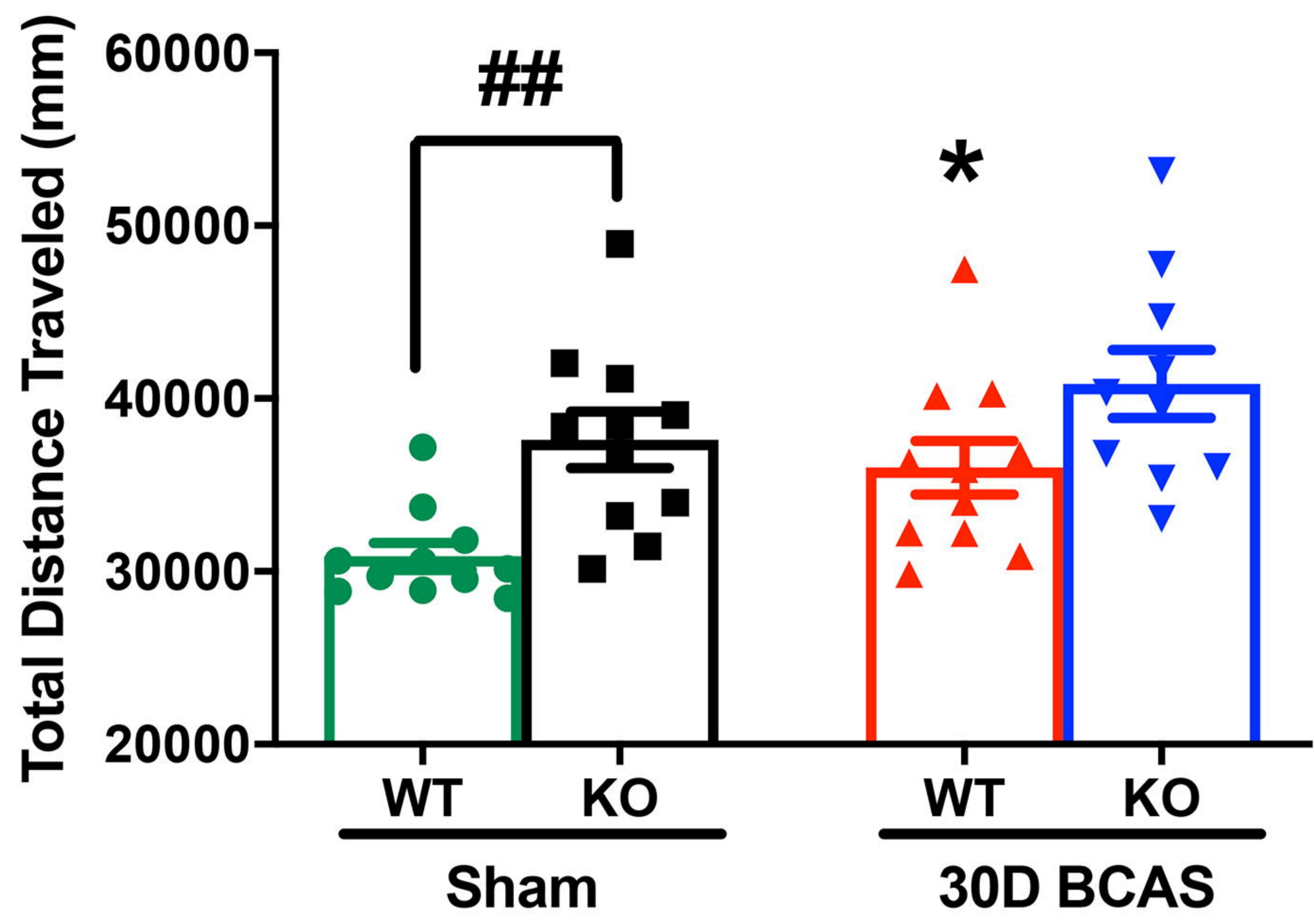
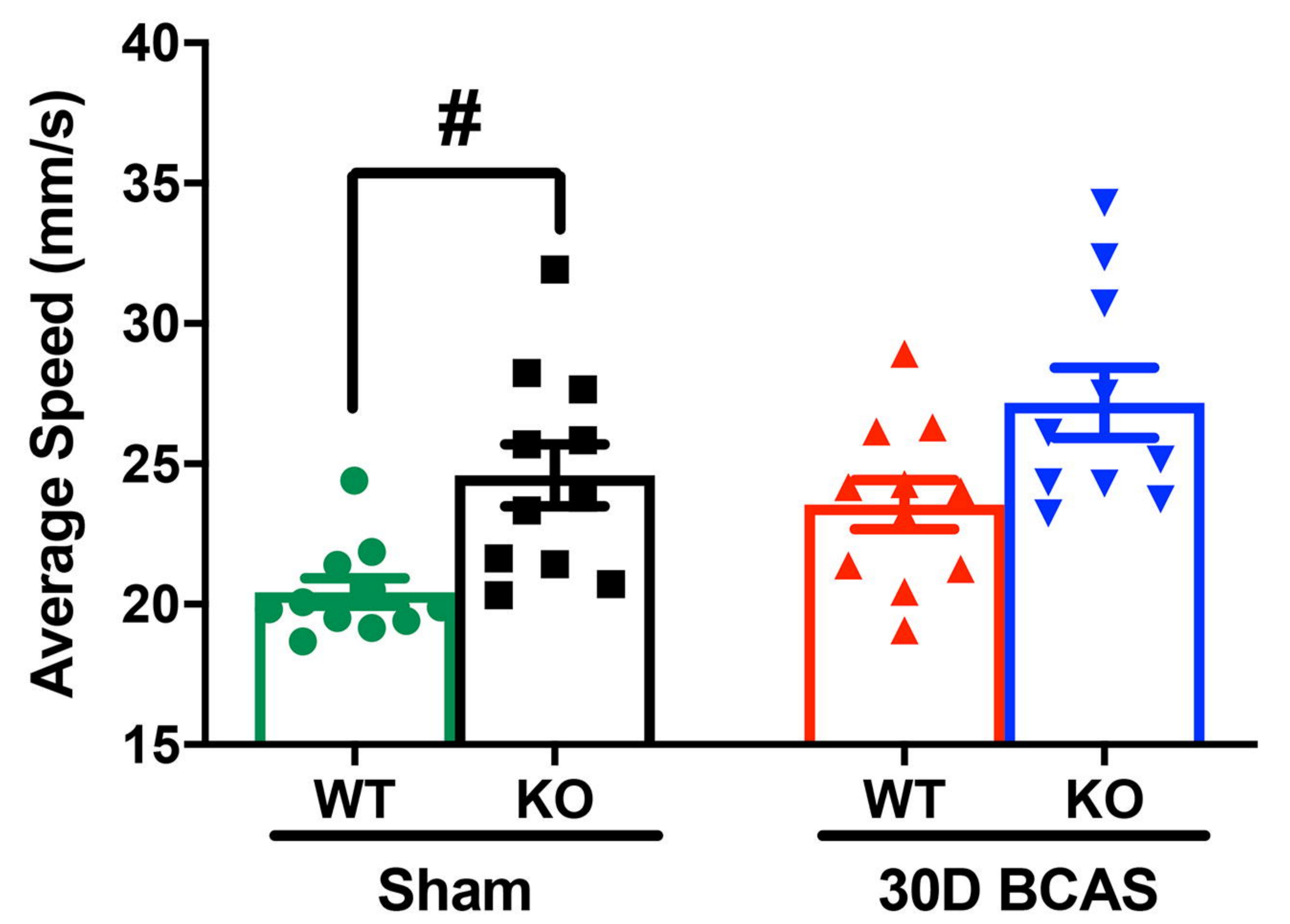
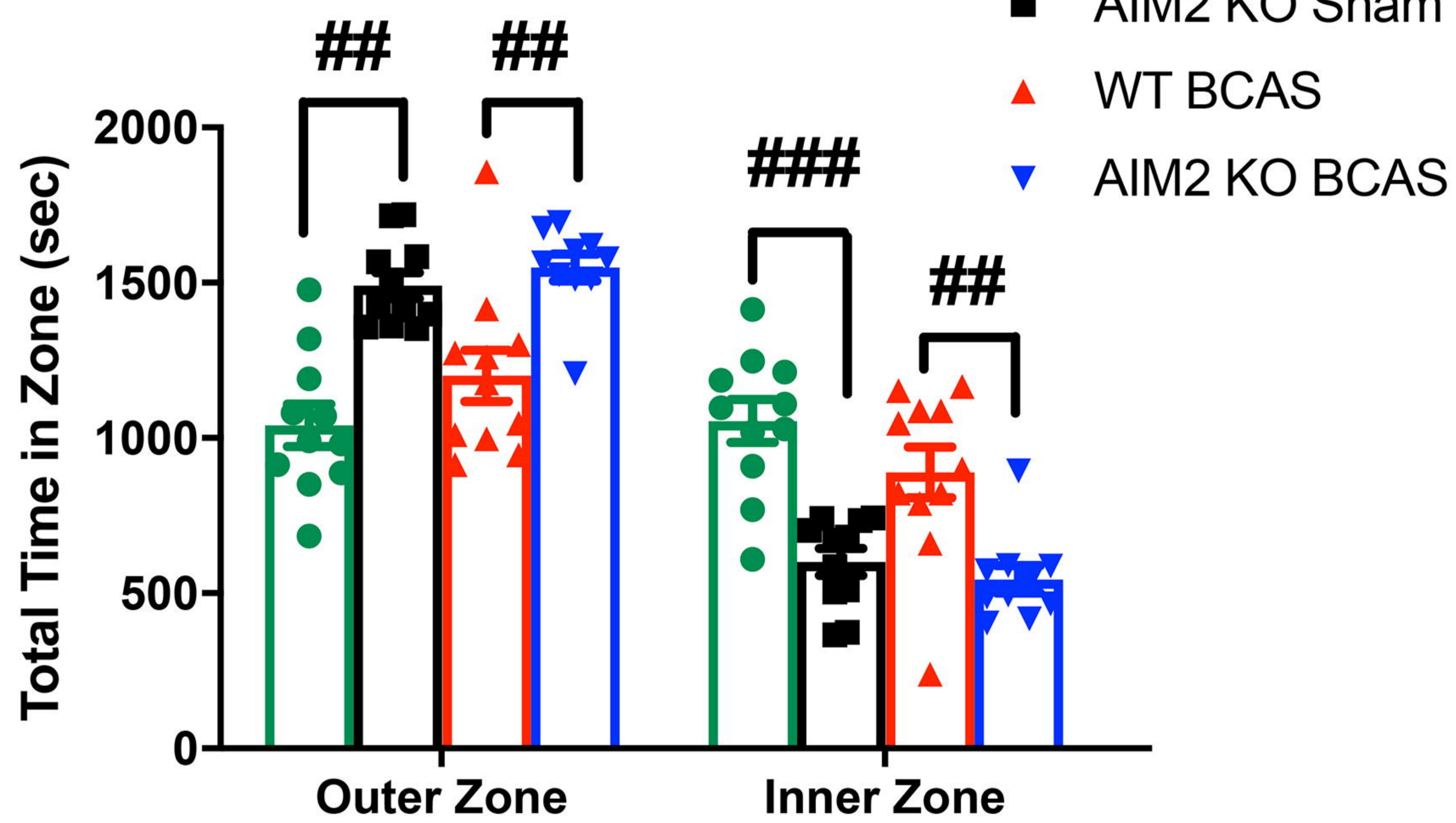
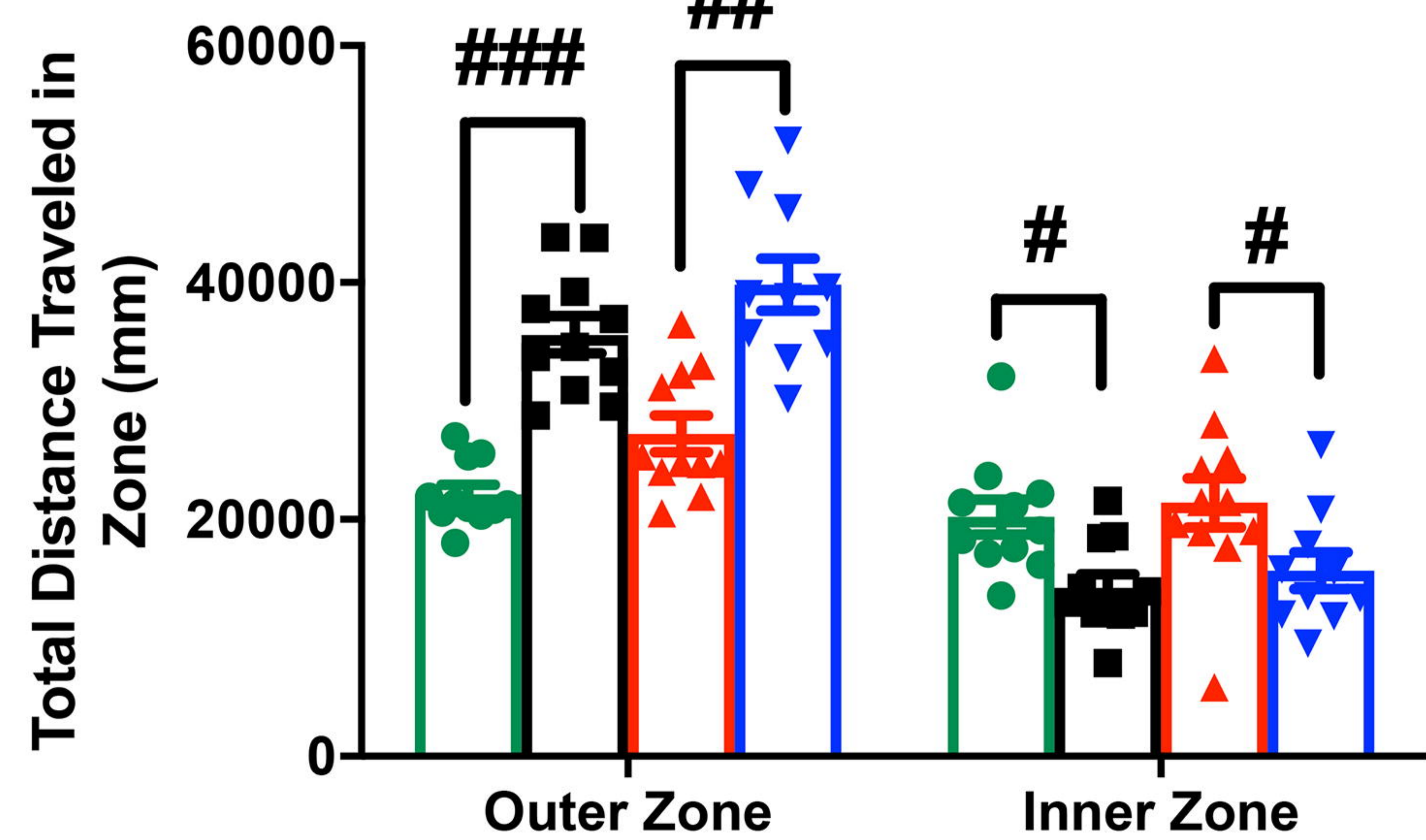
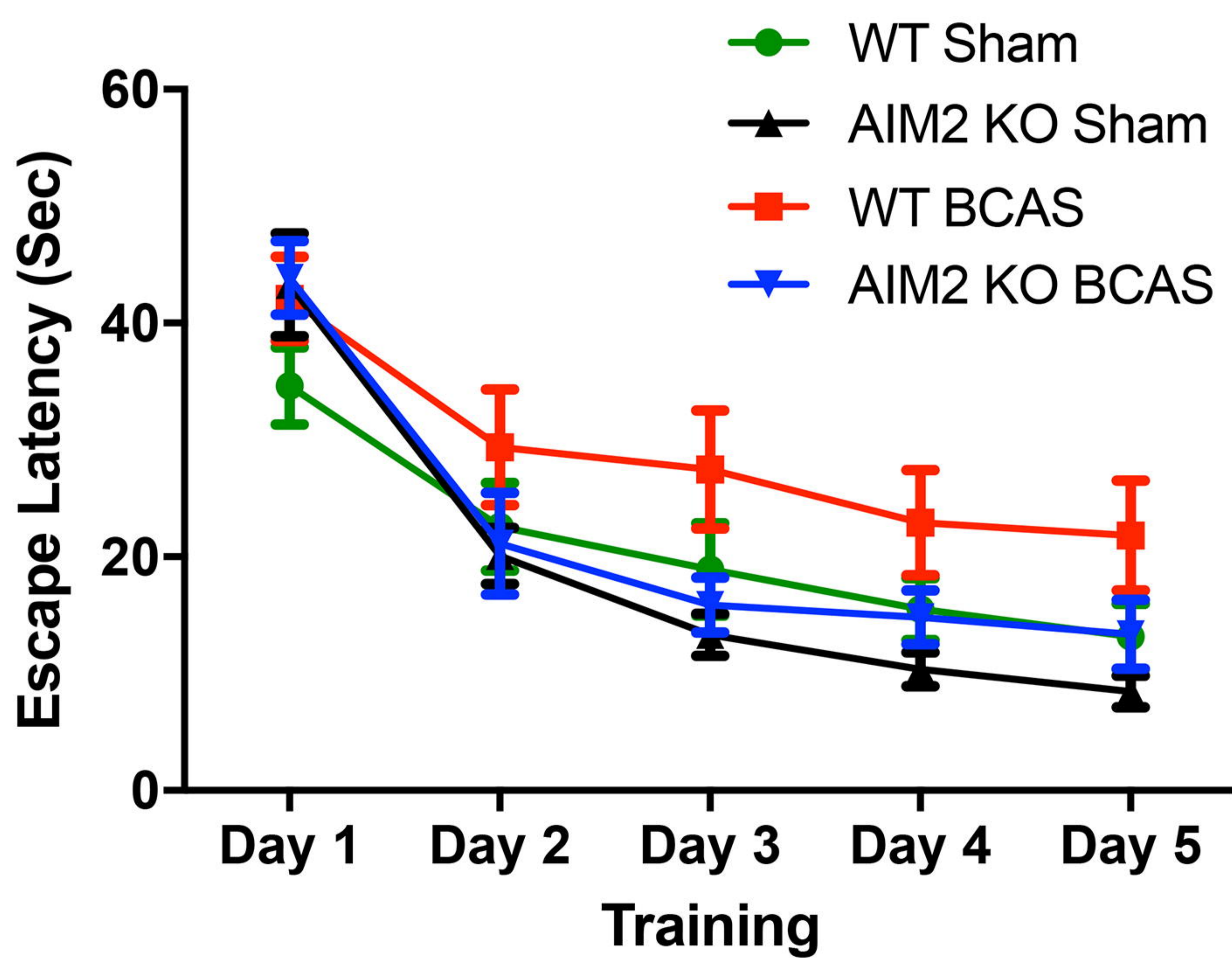
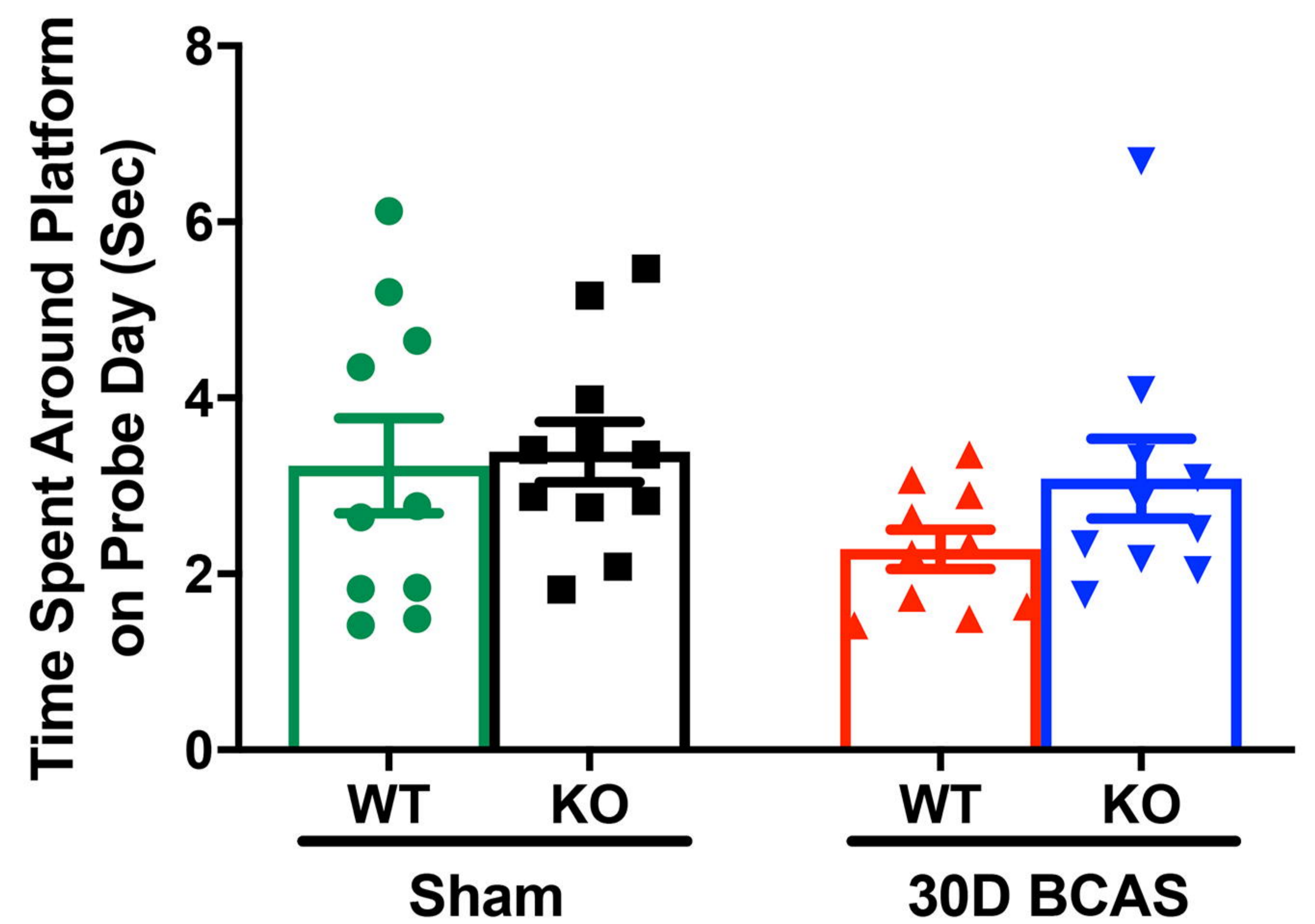
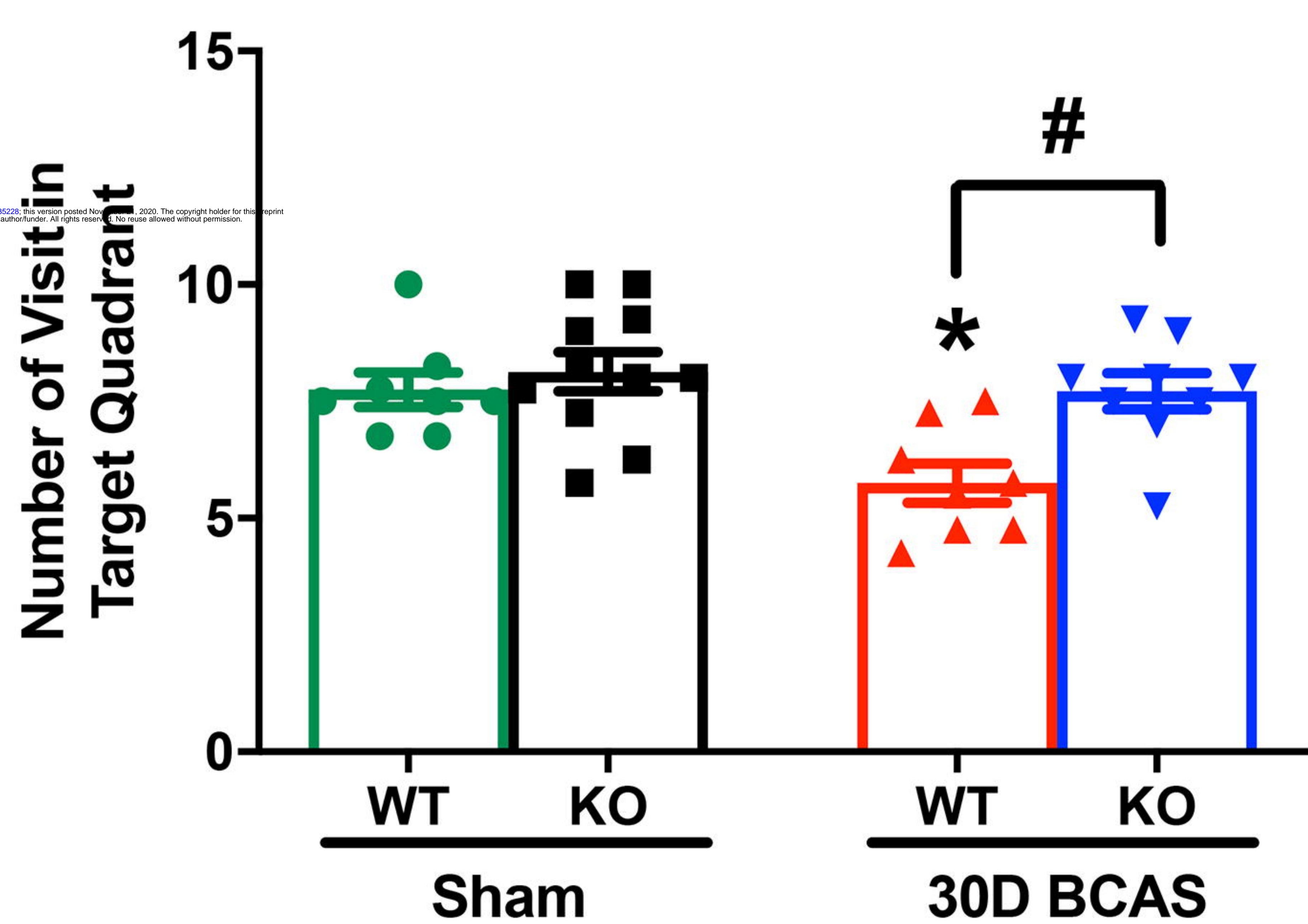










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