Gliogenesis from the subventricular zone modulates the extracellular matrix at the glial scar after brain ischemia

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ABSTRACT

Activation of the subventricular zone (SVZ) following cerebral ischemia is one of the brain's early responses to counteract neuron loss and minimize tissue damage. Impaired brain regions communicate with the SVZ through various chemotactic signals that promote cell migration and differentiation, primarily involving neural stem cells (NSC), neuroblasts, or glioblasts. However, the activation of gliogenesis and the role of newly formed astrocytes in the post-ischemic scenario remain subjects of debate. We have previously demonstrated that adenosine release after brain ischemia prompts the SVZ to generate new astrocytes. Here, we used transient brain ischemia in mice to identify the cellular origin of these astrocytes within the SVZ neurogenic niche and to investigate their role in the pathological process. By combining immunofluorescence, BrdU-tracing, and genetic cell labeling, we tracked the migration of newborn astrocytes, positive for the proteoglycan marker Thbs4, from the dorsal and medial SVZ to the perilesional barrier surrounding the ischemic core, known as the "glial scar". We found that these Thbs4-positive astrocytes modulate the dense extracellular matrix at the lesion border by both synthesizing and degrading hyaluronan. We also show that while the accumulation of this polymer at the lesion site is sufficient to recruit newborn astrocytes, its degradation at the SVZ correlates with gliogenesis. These findings suggest that newborn astrocytes could be a promising pharmacological target for modulating the glial scar after brain ischemia and facilitate tissue regeneration.

36 **INTRODUCTION**

37 Cell regeneration in the adult mammalian brain primarily occurs mainly in two neurogenic niches: the
38 subventricular zone (SVZ) in the lateral ventricle and the subgranular zone (SGZ) in the dentate gyrus
39 of the hippocampus (Bond et al., 2015). Adult brain stem cells are multipotent and originate from the
40 embryonic radial glia, thus sharing **key** cell markers and features with resident astrocytes. In the adult
41 mouse brain, neuroblasts from SVZ migrate through the rostral migratory stream (RMS) to the olfactory
42 bulb (OB), where neurogenesis takes place (Luskin, 1993). Neuronal hyperactivity (e.g., during
43 epilepsy), as well as pathological conditions (e.g., stroke) or natural (e.g., aging) processes, can impair
44 the physiological functions of the neurogenic niches, altering their neurogenic and gliogenic potential
45 (Chaker et al., 2016; Kralic et al., 2005; Sundholm-Peters et al., 2005). For instance, it is known that
46 SVZ cells are activated *in vivo* after focal ischemia following a middle cerebral artery occlusion
47 (MCAO) (Ceanga et al., 2021).

48 We have previously demonstrated in oxygen and glucose-deprived (OGD) organotypic brain slices that
49 the ischemic region and the SVZ establish a biochemical interplay based on a gradient of toxic and
50 protective factors released early after the insult (Cavaliere et al., 2006). Furthermore, others have
51 demonstrated that brain stroke models in mice can activate astrogliogenesis at the expense of
52 neurogenesis (Benner et al., 2013; Laug et al., 2019; Li et al., 2010). Benner and colleagues identified
53 a SVZ-derived astrocyte population characterized by high levels of the membrane-bound proteoglycan
54 thrombospondin 4 (Thbs4), a cell-cell and cell-matrix adhesion molecule that has been considered
55 neuroprotective against ischemic damage (Benner et al., 2013; Laug et al., 2019). Ablation of Thbs4 in
56 KO mice also produced abnormal ischemic scar formation and increased microvascular hemorrhage,
57 suggesting a role of SVZ astrogliogenesis in brain damage and glial scar formation induced by ischemic
58 stroke (Benner et al., 2013).

59 The perilesional barrier known as the *glial scar* assembles early after brain ischemia, isolating the
60 damaged area and limiting both the spread of pathogens and potentially toxic damage-associated
61 molecular patterns (DAMPs) into the surrounding area (Anderson et al., 2016; Bush et al., 1999;

62 Faulkner et al., 2004). Consisting of packed reactive astrocytes and a dense extracellular matrix
63 composed mostly of hyaluronic acid (HA), the glial scar represents a physical barrier for the SVZ-
64 generated newborn neurons that migrate into the ischemic core (Arvidsson et al., 2002; Yamashita et
65 al., 2006). Thus, modulating glial scar formation after brain ischemia may be a strategy to reduce tissue
66 damage and restore brain functionality. In ischemic stroke, the brain extracellular matrix (ECM) goes
67 through a significant reorganization due to injury and must undergo successful remodeling to promote
68 brain repair. ECM elements involved in the ischemic cascade induce activation of astrocytes and
69 microglia, which modulate ECM structure by releasing glycoproteins into the extracellular space
70 (Adams and Gallo, 2018; Fawcett, 2015). This crosstalk culminates in the formation of the glial scar,
71 which is created locally by reactive astrocytes and astrocytes-secreted ECM molecules such as HA and
72 chondroitin sulphate proteoglycans (Bush et al., 1999; Lau et al., 2013).

73 We have previously demonstrated that high extracellular adenosine levels, a hallmark of ischemic
74 insults, induce astrogliogenesis from the SVZ (Benito-Muñoz et al., 2016). Following our previous
75 results, here we characterize the SVZ response to brain ischemia and investigate the role of newly
76 generated astrocytes in the modulation of the glial scar after MCAO. We show that ischemic conditions
77 increase the number of newly generated astrocytes in the SVZ and their migration to the ischemic area.
78 We also propose the role of these astrocytes in modulating the extracellular matrix at the glial scar and
79 suggest this astrocyte population as a possible target for brain repair after brain ischemia.

80

81 **RESULTS**

82 **Thbs4 is expressed in the neurogenic niche of the SVZ.**

83 As suggested by others (Benner et al., 2013; Girard et al., 2014; Pous et al., 2020), Thbs4 is highly
84 expressed in astrocytes of the SVZ and RMS and might play a role in ischemia-induced neurogenesis.
85 To confirm these observations, we labeled the mouse SVZ by immunofluorescence for Thbs4. Thbs4
86 was mainly expressed in the SVZ, RMS, and OB but also in the cerebellum and ventral tegmental area

87 (Fig. 1A). In the SVZ, the staining was associated with cells with different morphologies (Fig. 1B),
88 ranging from cells with small primary apical processes in contact with the ventricle, to rounded NSC-
89 like cells in the dorsolateral SVZ horn. After quantification by direct count of Thbs4-positive cells,
90 Thbs4-positive astrocytes represented 21.4% of the total SVZ cells (Fig. 1C), with more than 60% of
91 them displaying a radial glia-like type-B morphology (Bond et al., 2015; Doetsch et al., 1999) (Fig.
92 1D). This is in accordance with other studies reporting Thbs4 expression in B-cells, either by
93 immunohistochemistry (Beckervordersandforth et al., 2010; Benner et al., 2013) or single-cell RNA
94 sequencing (Basak et al., 2018; Cebrian-Silla et al., 2021; Llorens-Bobadilla et al., 2015).

95 To validate the immunohistochemistry protocol for Thbs4, we labeled the SVZ cells with green
96 fluorescent protein (GFP) under the control of the Thbs4 promoter (pThbs4-eGFP) by postnatal (P1)
97 electroporation (Fig. 1E). Twenty-four hours after electroporation, almost 20% of total cells in the
98 dorsal SVZ expressed GFP (Fig. 1F), with a greater expression in the rostral SVZ (Fig. 1G), confirming
99 that Thbs4-positive cells were mainly localized in the neurogenic niche of the SVZ. This also suggests
100 that Thbs4 is expressed in NSCs at early postnatal stages, generating astrocytes alongside development.

101

102 **Thbs4 expression in the SVZ increases after brain ischemia**

103 To investigate whether brain ischemia could activate astrogliogenesis in the SVZ, we analyzed cell
104 proliferation and Thbs4 expression in the SVZ after MCAO. Sixty minutes of MCAO (Fig. S1A) caused
105 neuronal damage in the cortex and striatum, as observed by cresyl violet staining (Fig. S1B) and NeuN
106 immunofluorescence (Fig. S1C). The survival rate at 28 days post-lesion was around 50% (Fig. S1D).
107 Moreover, motor deficits and animal weight of ischemic mice were monitored every day after MCAO
108 for 30 days. Motor deficits improved overtime, while body weight dropped sharply the first 3 days after
109 MCAO and was partially restored at 30 dpi (Fig. S1E-F).

110 To study cell proliferation in the SVZ, we administered 50 mg/kg BrdU i.p. every two hours (3 doses
111 in total) the day before MCAO (Fig. S2A). We observed an increase in the number of total SVZ BrdU-

112 positive cells 24h after MCAO (Fig. S2B-C). Ki67 immunofluorescence of SVZ wholemount
113 preparations (Fig. S2D) also revealed a more than tenfold increase of proliferative cells 24h after
114 MCAO (Fig. S2E). In addition, we observed a significant increase in cleaved Caspase3-positive cells
115 24h post-lesion (Fig. S2F-G). These results suggest a fast and specific proliferative response in the
116 ischemia-induced SVZ.

117 We next analyzed Thbs4 staining in combination with Nestin, an NSC marker, and Doublecortin
118 (DCX), a marker of neuroblasts, before and after MCAO. SVZ was analyzed in sham and ischemic
119 mice 7-, 15- and 30-days post-ischemia (dpi). The immunofluorescence analysis of the SVZ (Fig. 2A)
120 showed a transient decrease in Nestin and DCX-positive cells but an increase of Thbs4-positive
121 astrocytes up to 15 dpi (Fig. 2B-D) compared to sham animals. The increase of Thbs4 astrocytes was
122 also confirmed by Western blot analysis using SVZ homogenates (Fig. 2E-F), evidencing a 2-fold
123 increased expression of Thbs4 in the ischemic SVZ. Similarly to immunofluorescence, Western blot
124 also showed decreased Nestin protein levels at 15 dpi (Fig. 2G). These results demonstrate that Thbs4
125 expression in the SVZ increases after ischemia, coinciding with a decrease in neuroblast markers,
126 suggesting that the classical pro-neuroblast program of the SVZ is interrupted in favor of the generation
127 of astrocytes after ischemia.

128

129 **Characterization of ischemia-induced cell populations in the SVZ**

130 To characterize the different cell populations of the SVZ activated after focal ischemia, we labeled the
131 whole pool of proliferating cells with 1% BrdU in drinking water for 14 days before MCAO (Codega
132 et al., 2014). One month after MCAO, BrdU in the SVZ primarily stained slow proliferative cells like
133 type B cells, whereas in rapid transit-amplifying cells (type C), BrdU was diluted away. Therefore, we
134 chose to label type C cells by intraperitoneal administration (50 mg/kg) of 5-Iodo-2′-deoxyuridine (IdU)
135 24h before euthanasia (Fig. 3A). To identify the cellular origin of newborn astrocytes, we analyzed the
136 SVZ 30 dpi by immunofluorescence co-labeling Thbs4 and GFAP together with BrdU or IdU. We
137 found a 2-fold increase in the total amount of proliferative cells (BrdU-positive) after MCAO with

138 respect to the sham group (Fig. 3B), whereas we did not observe a significant change in the number of
139 IdU-positive cells (Fig. 3C). The number of slow proliferative NSC (Thbs4/GFAP/BrdU) increased
140 linearly after MCAO (Fig. 3D, E) especially in the dorsal region of the SVZ (Fig. S3A), whereas the
141 number of activated NSC (Thbs4/GFAP/IdU) did not change significantly, either in the whole SVZ
142 (Fig. 3F) or in any subregion analyzed (Fig. S3B). These results suggest that, after ischemia, Thbs4-
143 positive astrocytes derive from the slow proliferative type B cells.

144

145 **Ischemia-induced newborn astrocytes migrate from the dorsal SVZ to ischemic regions**

146 To confirm that Thbs4-positive astrocytes originate from type B cells of dorsal SVZ, we labeled NSCs
147 of dorsal SVZ by electroporating the pCAGGSx-CRE plasmid in P1 Ai14 Rosa26-CAG-tdTomato
148 transgenic mice (Fig. 4A). Plasmid electroporation induced the expression of tdTomato (tdTOM) by
149 Cre recombination, enabling the spatial tracking and cell fate assessment of electroporated NCSs from
150 the dorsal SVZ. Three to four months after electroporation, we performed the MCAO, and tdTOM
151 fluorescence was analyzed at 7, 30, and 60 dpi. To confirm the efficient labeling of NSCs by the
152 electroporation protocol, we followed the tdTOM fluorescence in the OB (Fig. S4A, see methods). Only
153 those animals with tdTOM-positive cells in the OB were taken into account for later analysis. After
154 quantification of tdTOM-positive NSCs 7, 30, and 60 dpi, we observed a significant decrease in tdTOM-
155 positive cells in ischemic animals in all SVZ regions analyzed (Fig. 4B-C and Fig S4B), suggesting that
156 they either left the area, or died. Conversely, there was a general increase of tdTOM-positive cells
157 outside the SVZ (Fig. 4D-E), especially in the intermediate and caudal axis of the whole brain compared
158 to sham animals (Fig. S4D-G), suggesting that electroporated cells had migrated away from the SVZ.

159 To identify the proportion of NSCs that differentiated into Thbs4-positive astrocytes following
160 ischemia, we analyzed the co-expression of Thbs4 with tdTOM after MCAO. Despite the general
161 decrease of tdTOM-positive cells in the SVZ, the fraction of Thbs4/tdTOM cells increased at 30 dpi
162 after MCAO (Fig. 4F-G), reaching a maximum increase at 60 dpi (Fig. 4H), in particular in rostral and
163 intermediate SVZ (Fig. S4C). Finally, we analyzed the Thbs4-positive astrocytes recruitment to the

164 ischemic regions. More than half of the total tdTOM-positive cells in the infarcted area expressed the
165 Thbs4 marker at 60 dpi (Fig. 4I-J). These results suggest that ischemia-induced astrogliogenesis in the
166 SVZ occurs in type B cells from the dorsal region and that these newborn Thbs4-positive astrocytes
167 migrate to the ischemic areas.

168

169 **Thbs4-positive astrocytes accumulate at the glial scar after brain ischemia.**

170 Besides its role in the neurogenic niches, Nestin is also rapidly and locally expressed by reactive
171 astrocytes and participates in glial scar formation (Frisén et al., 1995). Thus, we used Nestin
172 immunofluorescence as a reliable tool to identify the lesion border where the glial scar occurs (Fig. 5A).
173 Nestin staining of the scar was also compared with more classical methodologies like TTC staining
174 (Fig. 5B), where viable tissue is stained red and dead tissue is left unstained. The white-pale pink area
175 was then measured as infarct tissue by TTC staining (Fig. 5C). Unlike the TTC staining, which can only
176 be performed shortly after the lesion, the visualization of the glial scar with Nestin immunofluorescence
177 allowed us to analyze the lesion at later time-points. We observed a rapid formation of the glial scar as
178 early as 7 dpi (Fig. 5D). Analyzing the expression of the two astrocytic markers, Thbs4 and GFAP, as
179 a percentage of the glial scar region-of-interest (ROI), we observed different dynamics and localization.
180 GFAP expression increased rapidly after 7 days within the glial scar ROI, whereas Thbs4 was
181 upregulated at 15 dpi (Fig. 5E). This delay suggested that the glial scar harbors two different astrocytic
182 sub-populations: GFAP-only or GFAP/Thbs4. To analyze the spatial location of GFAP and Thbs4
183 markers within the Nestin border, we measured the fluorescence intensity profile of damaged area at 30
184 dpi. GFAP was found in both the scar and core areas, whereas Thbs4 fluorescence was found mostly at
185 the borders of the glial scar (Fig. 5F-G). Analysis and dynamics of Thbs4-positive astrocyte migration
186 were further performed in the corpus callosum (CC), striatum (Str), cortex (Ctx) and olfactory bulb
187 (OB) (Fig. S5A). We observed a linear increase of Thbs4-positive astrocytes from 7 to 60 dpi, both in
188 the Ctx and Str (Fig. S5B). Migration occurred mainly through the corpus callosum; among the total
189 GFAP-positive astrocytes, 36.5% were also positive for Thbs4, suggesting that more than one-third of

190 the astrocytes in the glial scar originated from the SVZ (Fig. S5C). Likewise, the number of DCX-
191 positive cells dramatically decreased 15 dpi in all OB layer (Fig. S5D-F) together with a significant
192 decrease of BrdU-positive cells (Fig. S5G) DCX/BrdU- positive neuroblasts in the RMS (Fig. S5H),
193 suggesting that ischemia induced a change in the neuroblasts' ectopic migratory pathway, depriving the
194 OB layers of SVZ newborn neurons.

195

196 **Thbs4-positive astrocytes modulate extracellular matrix homeostasis at the glial scar**

197 To investigate the role of Thbs4-positive astrocytes in the glial scar, we analyzed hyaluronic acid (HA),
198 the main component of the brain extracellular matrix (ECM), using the hyaluronic acid binding protein
199 (HABP) as a marker. Following ischemic damage, local astrocytes (GFAP+/Thbs4-) react by adopting
200 a fibrotic morphology, producing high-molecular weight HA (HMW-HA) to form the glial scar and
201 isolate the damaged area (Preston and Sherman, 2011). We then examined HA deposition in the corpus
202 callosum and the infarcted areas after MCAO. HABP immunofluorescence (Fig. 6A) and image
203 analysis revealed HA accumulation in the infarcted areas. Skeleton analysis, which quantifies the length
204 of HA chains and correlates with its molecular weight, identified progressively longer HA cable-like
205 structures at the glial scar as the infarct evolved (Fig. 6B). Fractal dimension analysis, which reflects
206 the interconnectivity of the extracellular matrix (Soria et al., 2020), further confirmed HA accumulation
207 and increased matrix complexity in infarcted areas, but not in corpus callosum (Fig. 6C), suggesting
208 that ECM accumulation is different in white and gray matter.

209 The proximity of Thbs4-positive astrocytes to HA in the ischemic scar (Fig. 6D-E) led us to investigate
210 if this population contributed to ECM production and, consequently, to scar formation. Since HA is
211 produced exclusively at the cell membrane (Sherman et al., 2015; Zimmermann and Dours-
212 Zimmermann, 2008), we analyzed HA labeling within 1 µm of the Thbs4-positive cell membrane in
213 high-resolution confocal images (Fig. 6F). Image analysis showed a significant increase in HABP area
214 at Thbs4-positive astrocyte-cell membranes after MCAO (Fig. 6G), suggesting a role of Thbs4-positive
215 astrocytes in HA production within the glial scar.

216 To explore the potential role of Thbs4-positive astrocytes in HA removal, we quantified intracellular
217 HABP spots in Thbs4-positive astrocytes at the glial scar. While HMW-HA cleavage occurs
218 extracellularly, HA fragments can be internalized for degradation by lysosomal hyaluronidases (Preston
219 and Sherman, 2011; Zimmermann and Dours-Zimmermann, 2008). Intracellular HABP spots were
220 compared between Thbs4-positive astrocytes and local astrocytes (GFAP-positive/Thbs4-negative)
221 (Fig. S6A). Thirty days after MCAO, Thbs4-positive astrocytes showed more intracellular HABP than
222 local astrocytes in the infarcted cortex (Fig. S6B). However, the number of HA spots internalized was
223 higher in local astrocytes compared with Thbs4 astrocytes (Fig. S6C), suggesting that Thbs4-positive
224 cells internalize HA in fewer vesicles. To confirm that astrocytes were degrading HA, we performed an
225 immunofluorescence analysis of CD44 (Fig. S6D). CD44 is a transmembrane glycoprotein receptor that
226 mediates HA internalization and degradation, and therefore ECM remodeling (Dzwonek and
227 Wilczynski, 2015). We detected that around 50% of the total Thbs4-positive astrocytes contained
228 internalized CD44 receptor, in the striatum 30 dpi (Fig. S6E), with higher numbers (60%) for the
229 infarcted corpus callosum. These findings suggest that Thbs4- positive astrocytes participate in both the
230 formation and degradation of the extracellular matrix at the glial scar.

231 To further investigate HA internalization by astrocytes, we conducted *in vitro* experiments using rat
232 SVZ neural stem cells cultures. An oxygen and glucose deprivation (OGD) protocol was applied as an
233 ischemia model and cells were fixed 7 days after OGD (Fig. S7A). HA was analyzed via HABP
234 immunofluorescence (Fig. S7B), both inside and outside the Thbs4-positive cells (Fig. S7C). Thbs4-
235 positive cells showed increased intracellular HA after OGD (Fig. S7D), while extracellular HA levels
236 decreased (Fig. S7E). However, this phenomenon was reversed after adding hyaluronidases in the
237 extracellular space (Fig. S7F-G), suggesting that Thbs4-positive cells from SVZ homogenates can
238 internalize or synthesize HA depending on external stimuli. HA internalization was also measured in
239 Iba1-positive cell cultures where we observed a reduced internalization compare to Thbs4-positive cells
240 (Fig. S7H). Lastly, metalloproteinase activity was found to be increased in Thbs4 cultures following
241 OGD (Fig. S7I). Altogether, these results suggest Thbs4-positive cells can internalize HA after ischemic
242 conditions but also synthetize it when ECM is degraded.

243

244 **Hyaluronan accumulation is sufficient but not exclusive for the recruitment of Thbs4-positive**
245 **astrocytes**

246 Since Thbs4-positive astrocytes migrate to the glial scar but not to the ischemic core, we hypothesized
247 that HA, the primary component of the ischemic scar, may be the trigger signal for recruiting Thbs4-
248 positive astrocytes. To test this hypothesis, we simulated the ECM accumulation that occurs at the scar
249 by overexpressing the Hyaluronan synthase 2 (HAS2) from the naked mole rat (*Heterocephalus glaber*),
250 a tool often used in mice to produces HA of very high molecular weight (Tian et al., 2013; Zhang et al.,
251 2023). We used a viral vector (AAV2/9-CAG-HAS2-3xFlag) with a FLAG tag to visualize transduced
252 cells after intracerebral inoculation in the striatum and checked HA accumulation in FLAG-positive
253 areas using HABP staining (Fig. 7A). We overexpressed HAS2 (or GFP as control) unilaterally. In a
254 subset of animals that underwent MCAO, AAV-HAS2 was injected contralateral to the infarct, to
255 compare Thbs4-positive astrocytes recruitment by both stimuli, the inflammation from the ischemic
256 damage and the HA accumulation *per se* (Fig. 7B). FLAG-positive cells were quantified as a surrogate
257 marker of viral infection for both control and MCAO groups (Fig. 7C). The GFP control group did not
258 increase HA production *per se* (Fig. 7D). However, HA overexpression was observed in HAS2 groups
259 (both control and MCAO group) by HABP immunofluorescence (Fig. 7E-F).

260 HA accumulation was sufficient to recruit the Thbs4-positive astrocytes from the ipsilateral SVZ 45
261 days after AAV-HAS2 inoculation (Fig. 7G), with a similar time window to that observed after MCAO.
262 Interestingly, when we induced a lesion by MCAO and performed viral overexpression of HAS2 in the
263 contralateral hemisphere, we found that Thbs4-positive astrocytes preferentially migrated to the
264 ischemic glial scar (Fig. 7G). This migration of Thbs4-positive astrocytes to the site of ischemic damage
265 occurred despite no differences in HA overexpression between contralateral (HAS2 infected) and
266 ipsilateral (infarcted area) hemispheres (Fig. 7H). These results suggest that the accumulation of HMW-
267 HA alone is sufficient to activate and recruit Thbs4-positive astrocytes from the SVZ. However, the

268 preferential migration to the ischemic scar indicates that brain ischemia generates a more potent
269 biochemical signal that not only activates the SVZ but also recruits newborn astrocytes.

270

271 **Ischemia-induced gliogenesis at the SVZ correlates with local hyaluronan degradation**

272 It is known that the niche microenvironment has a unique ECM composition (Kjell et al., 2020) and
273 that the stiffness of the niche plays a crucial role in the proliferation and differentiation of progenitor
274 cells (Long and Huttner, 2019; Segel et al., 2019). After observing the close association between Thbs4-
275 positive astrocytes and the ECM at the glial scar, we decided to study the Thbs4/HA tandem in the SVZ
276 following MCAO (Fig. 8A). We did not observe any significant changes in the HA area across the
277 entire SVZ but noted a decrease at 7 and 15 dpi in the dorsal SVZ (Fig. 8B-D). Skeleton analysis showed
278 fragmentation of HA exclusively in the dorsal SVZ at 7 and 15 dpi (Fig. 8E-G), i.e., earlier than the
279 migration of these newborn astrocytes toward the lesion.

280 Next, we examined the expression of genes related to HA degradation (*Hyase 1, 2* and *3*) and synthesis
281 (*HAS 1* and *HAS 2*). We also analyzed *Thbs4* and hypoxia-inducible factor 1-alpha (*Hif1a*), which are
282 known to be upregulated in the SVZ following brain ischemia (Benito-Muñoz et al., 2016; Liu et al.,
283 2007). Fresh SVZ tissue was collected from sham, 15 and 30 dpi mice (Fig. 8H). We observed an
284 increased expression of all Hyases at 15 dpi and slightly decreased by 30 dpi, whereas synthases (HAS)
285 showed a gradual increase from 15 to 30 dpi (Fig. 8I). Additionally, we observed an upregulation of
286 Thbs4 and Hif1a both at 15 and 30 dpi (Fig. 8I). These results suggest that HA degradation in the SVZ
287 is correlated with the Thbs4 response to ischemia.

288

289 **DISCUSSION**

290 The effects of brain insult on adult neurogenesis have been extensively discussed, both in animal models
291 and humans. However, whether these injuries can trigger the generation of newborn astrocytes from the

292 neurogenic niches remains a topic of debate. Neural stem cells in the adult brain are a remnant of
293 embryonic radial glia. Some researchers suggest that depletion of neurogenesis in the adult brain is a
294 consequence of astrogliogenesis (Encinas et al., 2011), while others propose astrogliogenesis is part of
295 a neurorestorative program originating from the neurogenic niches (Bonzano et al., 2018; Sohn et al.,
296 2015). Despite these differing perspectives, direct and definitive evidence of the generation of newborn
297 astrocytes from the SVZ or the SGZ has yet to be presented, as well as the potential role of these
298 astrocytes in aging and pathological conditions. In this study, we confirm that a strong and acute
299 damage-associated stimulus in the brain, such as an ischemic infarct, prompts astrogliogenesis in the
300 SVZ. Furthermore, we demonstrate that these astrocytes, which are positive for Thbs4, migrate to the
301 glial scar and play a role in its modulation, being capable of synthesis and degradation of hyaluronan,
302 a key component of the ECM at the scar.

303 Our findings align with previous studies, where cortical injury initiated by photothrombotic/ischemia
304 was shown to trigger a substantial production of Thbs4-positive astrocytes from the SVZ, a process
305 modulated by Notch activation (Benner et al., 2013). In this work, the authors suggested a clear role for
306 Thbs4-positive astrocytes in modulating the ischemic scar. In fact, homozygotic deletion of the Thbs4
307 gene led to abnormal glial scar formation and increased microvascular hemorrhage following brain
308 ischemia. Similarly, our results demonstrate that the adult neurogenic niche at the SVZ responds early
309 after ischemic stroke, degrading local hyaluronan plausibly to modify niche stiffness and facilitate
310 proliferation and differentiation of NSCs. Further experiments using Thbs4-knockout models will help
311 confirm this hypothesis.

312 By labeling NSC via *in vivo* postnatal electroporation, we provided proof of the origin and migration
313 of newborn Thbs4-positive astrocytes from the SVZ to the ischemic scar in response to the injury.
314 Notably, Thbs4 is expressed in the newborn astrocytes that migrate to the scar, whereas local reactive
315 astrocytes at the site of injury express only GFAP. It is likely that SVZ-derived newborn astrocytes
316 require Thbs4 expression to facilitate their migration to the ischemic area, as Thbs4 deletion has been
317 shown to impair migration of SVZ-derived cells along the RMS (Girard et al., 2014).

318 It is noteworthy that while the local, “GFAP-only”, reactive astrocytes at the scar are capable of forming
319 the required ECM (Cregg et al., 2014; Wanner et al., 2013), we show that Thbs4-positive (Thbs4/GFAP)
320 astrocytes are also recruited from the SVZ to modulate scar formation. Unlike local reactive astrocytes,
321 which are hastily generated and present both at the core and periphery of the lesion, the newborn
322 astrocytes from the SVZ arrive later to the injury. We demonstrate that these Thbs4-positive astrocytes
323 migrate from the SVZ to the lesion and halt at the lesion border (precisely defined by Nestin staining),
324 establishing residence at the scar but not beyond.

325 The molecular signals that trigger gliogenesis in the SVZ and recruit newborn astrocytes to the lesion
326 remain unclear. We previously identified adenosine, a byproduct of ATP hydrolysis, as a strong
327 candidate, as it stimulates astrogliogenesis through A1 receptors following OGD *in vitro* and ischemia
328 *in vivo* (Benito-Muñoz et al., 2016). Here, we show that viral-mediated overproduction of HA, the
329 primary component of the glial scar, is sufficient to activate the generation and migration of the Thbs4-
330 positive astrocytes from the SVZ. However, our findings also show that the ischemic infarct, with its
331 proinflammatory (cytokines, chemokines) and proteolytic (MMPs, ROS) signals (Jayaraj et al., 2019),
332 serves as a more potent attractor than the ECM alone.

333 The glial response from the SVZ we propose in this study can have several functional implications.
334 Thbs4-positive astrocytes generated from the SVZ might act as a second wave of reactivity, supporting
335 local astrocytes in modulating ECM formation. Hyper-reactive astrocytes at the lesion border decrease
336 over time, along with reductions in HA synthesis and CD44 activity, suggesting that the second wave
337 of Thbs4-positive astrocytes from the SVZ could sustain high levels of HA production (Lindwall et al.,
338 2013). Thus, Thbs4-positive astrocytes may be crucial during this delayed temporal window when glial
339 scar remodeling is essential for neurological recovery (Cai et al., 2017; Dzyubenko et al., 2018).
340 However, it's important to note that only about one-third of the astrocytes at the glial scar are derived
341 from the SVZ. This suggests that while SVZ-derived astrocytes may be important for glial scar
342 maintenance, local astrocytes remain the central players in this process.

Based on our observations of both HA production and internalization by Thbs4-positive astrocytes *in vivo* within the ischemic region and *in vitro* following OGD, we propose a role for these astrocytes in the modulation, and possibly maintenance, of the ECM at the scar. It is likely that ECM degradation at the scar is also carried out by professional macrophages, such as microglia, which are known to be recruited to the scar (Fawcett and Asher, 1999) and have been shown to degrade ECM through phagocytosis (Nguyen et al., 2020; Soria et al., 2020). Understanding which cells within the scar environment participate in its modulation, particularly its degradation, holds highly therapeutic value for promoting regeneration (Dzyubenko et al., 2018; Silver and Miller, 2004). Here, we shed light on the unknown role of SVZ-derived astrocytes activated by ischemic brain injury and propose them as critical players in tissue regeneration after brain ischemia.

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AUTHOR CONTRIBUTIONS

M.A. conducted experiments and collected and analyzed data. M.C.T. and H.C. provided the Ai14-Rosa26-CAG-tdTomato$^{flx/flx}$ mice and technical input for electroporation. B.D. provided the AAV-HAS2 and conceived the idea of glial scar simulation. F.C., F.N.S. and C.M. secured funding. C.M. provided infrastructural support and scientific feedback. F.C. and F.N.S. conceived and supervised the study. F.P.C. supervised the study. M.A. and F.N.S. prepared the figures. M.A., F.N.S. and F.C. wrote the paper, with input from all authors.

METHODS

Animals

Male and female wild-type C57BL6/J mice and Ai14-Rosa26-CAG-tdTomato$^{flx/flx}$ transgenic mice bred at the animal facility of UPV/EHU were used for *in vivo* experiments. Sprague Dawley rat pups from P4 to P7 were used for *in vitro* experiments. All animals for *in vitro* and *in vivo* studies were handled in accordance with the European Communities Council Directive (2010/63/EU). All possible efforts were made to minimize animal suffering and the number of animals used. Animals were maintained under standard laboratory conditions with food and water *ad libitum*.

Transient MCAO

Brain ischemia was induced in 3-4-month old C57BL/6 mice (approximately 25 g) by occluding 60 minutes of the lenticulo-striate arteries with a 10 mm length of silicone-coated 6-0 monofilament nylon suture (602223PK10, Doccol Corporation) using a modification of the protocol described by (Gelderblom et al., 2009). Arteries occlusion generated a specific lesion in the cortex and striatum that was evaluated at different times after the MCAO (7, 15, 30 or 60 days after occlusion). Ischemic damage was evaluated in the ipsilateral hemisphere by Cresyl violet staining, Nestin immunofluorescence or hyaluronan staining. Sham animals, in which arteries were visualized but not disturbed, were used as an experimental control. Both experimental groups, sham and ischemic, were anesthetized with a mix of isoflurane and oxygen (induction of anesthesia with 4% isoflurane and maintenance for surgery at 1.5% isoflurane) and maintained under analgesia with buprenorphine 24 hours after ischemia (0.03 mg/Kg body weight i.p. every 12h). Animal wellness was evaluated daily, controlling the body weight and the motor deficit of the animal by means of neurological scores and pole test. Motor deficit was assessed in each animal 1h after MCAO and later every 24h. Animals without neurological deficits were excluded from the study.

401 **5-bromo-2'-deoxyuridine (BrdU) protocol**

402 The total pool of proliferating cells was labelled with three injections of 50 mg/kg 5-bromo-2'-
403 deoxyuridine (BrdU) the day before MCAO protocol. Animals were sacrificed the day after MCAO to
404 analyse acute SVZ proliferation. Chronic BrdU protocol was performed for quiescent NSC
405 identification (Codega et al., 2014). Briefly, 1% of BrdU was administered in the drinking water for 14
406 days. On day 15, BrdU was removed and animals were subjected to MCAO protocol. Mice were
407 sacrificed and perfused at 3, 7, 15, and 30 days after MCAO, although only animals with a temporal
408 window of 30 days between MCAO and perfusion were used for statistical analysis. Activated cells
409 were labelled by i.p. injection of 50 mg/Kg 5-iodo-2′-deoxyuridine (IdU) 24 hours before euthanasia.

410

411 **Electroporation**

412 Ai14-Rosa26-CAG-tdTomato$^{flx/flx}$ transgenic mice and pCAGGS-CRE plasmid were kindly provided
413 for Prof. Harold Cremer and Marie-Catherine Tiveron from Institut de Biologie du Développement de
414 Marseille (IBDM). Postnatal electroporation was performed as described previously (Platel et al., 2019).
415 Mice pups from P0-P2 were anesthetized by hypothermia. pCAGGS-CRE plasmid was used at a final
416 concentration of 2.5 µg/ul and 2µl was injected in the lateral ventricle by expiratory pressure using a
417 glass micropipette. Following injection, pup brains were placed between electrodes targeted the dorsal
418 SVZ and subjected to five 95V electrical pulses (50 ms, 950 ms intervals). BTX ECM399 (Thermo
419 Fisher Scientific) electroporator was used with 7 mm tweezer electrodes. Electroporated pups were
420 reanimated at 37ºC before returning to the mother.

421 In order to analyze the Thbs4 positive cells in the SVZ, we subcloned the eGFP gene (from a pCMV-
422 eGFP N1) into a PGL3-basic backbone containing mouse Thbs4 promoter, kindly provided by Stenina-
423 Adognravi lab (Muppala et al., 2017). pThbs4-GFP was also electroporated in P0-P2 mice, and
424 euthanized 1-day post-electroporation to corroborate Thbs4 expression in the SVZ at postnatal stages.

425

426 **AAV vector production**

427 AAV2/9-CAG-HAS2-3xFlag vector was produced by polyethylenimine (PEI) mediated triple
428 transfection of low passage HEK-293 T/17 cells (ATCC; cat number CRL-11268). The respective AAV
429 expression plasmid expression plasmid pNMRHAS2_N1 (provided by Dr Vera Gorbunova) was
430 cotransfected with the adeno helper pAd Delta F6 plasmid (Penn Vector Core, cat # PL-F-PVADF6)
431 and AAV Rep Cap pAAV2/9 plasmid (Penn Vector Core, cat # PL-TPV008). AAV vector was purified
432 as previously described (Bourdenx et al., 2015). Cells are harvested 72 h post-transfection, resuspended
433 in lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 8.5) and lysed by 3 freeze-thaw cycles (37°C/-
434 80°C). The cell lysate is treated with 150 units/ml Benzonase (Sigma, St Louis, MO) for 1 h at 37°C,
435 and the crude lysate is clarified by centrifugation. Vectors are purified by iodixanol step gradient
436 centrifugation and concentrated and buffer-exchanged into Lactated Ringer's solution (Baxter,
437 Deerfield, IL) using vivaspin20 100 kDa cut-off concentrator (Sartorius Stedim, Goettingen, Germany).
438 Titrations were performed at the transcriptome core facility (Neurocentre Magendie, INSERM U862,
439 Bordeaux, France). The genome-containing particle (gcp) titer was determined at a concentration of
440 1.10^{13} gcp/ml by quantitative real-time PCR using the Light Cycler 480 SYBR green master mix
441 (Roche, cat # 04887352001) with primers specific for the AAV2 ITRs (fwd 50-
442 GGAACCCCTAGTGATG GAGTT-3'; rev 50-CGGCCTCAGTGAGCGA-30) on a Light Cycler 480
443 instrument. The purity assessment of vector stocks was estimated by loading 10 µl of vector stock on
444 10 % SDS acrylamide gels; total proteins were visualized using the Krypton Infrared Protein Stain
445 according to the manufacturer's instructions (Life Technologies).

446

447 ***In vivo* AAV-HAS2 injections**

448 Mice were anesthetized with isoflurane and fixed to a stereotaxic frame connected with a gas mask. We
449 injected 1µl of adeno-associated virus (AAV2/9) CAG-HAS2-3xFlag (1x10^13 gc/µl) in the
450 contralateral striatum (AP: +1.0; ML: -1.8; DV: -3.2) to simulate scar tissue hyaluronan. After two
451 weeks, the ischemic group was subjected to the MCAO model in the ipsilateral hemisphere and perfused

452 30 days later. Only slices with representative lesions in both hemispheres were used for image and
453 statistical analysis.

454

455 **Immunofluorescence**

456 Mice were anesthetized with an intraperitoneal injection of pentobarbital (100 mg/kg). Brain tissues
457 were fixed by intracardiac perfusion using 4% paraformaldehyde. Fixed brains were sliced at 40 µm of
458 thickness using the Leica VT1200S vibratome (Leica Microsystems) and subjected to
459 immunofluorescence. For standard immunofluorescence protocol, tissue slices were permeabilized with
460 0.1% Triton and non-specific epitopes blocked with 1% Bovine Serum Albumin and 10% Donkey
461 serum in PBS. Primary antibodies (Supplementary Table 1) were incubated at different concentrations
462 (see Table 1 for specification) overnight at 4ºC and then washed three times with 0.1% Triton in PBS.
463 Secondary conjugated antibodies were incubated for 1 h at room temperature. After three washes with
464 0.1% Triton in PBS, cells were stained for 10 min with DAPI and further washed with PBS. Finally,
465 coverslips were mounted with Fluoromount (SouthernBiotech) and analyzed by fluorescence using the
466 confocal microscope Leica TCS SP8. For hyaluronic acid labeling, endogenous biotins were pre-
467 blocked using Streptavidin/Biotin blocking solution (Vector Labs, #SP-2002) following the
468 manufacturer's instructions and further blocked with PBS 1X 0.1% Saponin 1% BSA for one hour at
469 room temperature. Primary antibodies and biotinylated Hyaluronic Acid Binding Protein (HABP,
470 #385911 Millipore) were incubated for 72 hours at 4°C or 24 hours at room temperature. After three
471 washes with PBS, 1X 0.1% Saponin, secondary antibody and DAPI diluted in the same blocking buffer
472 were incubated for one hour at room temperature. Slices were mounted with Mowiol (Millipore
473 #475904) in superfrost slides (Thermo Fisher Scientific) and #1.5 coverslips.

474

475 **Imaging and image analysis**

476 Confocal images were acquired in a Leica TCS SP8 with 20x (0.8 NA) and 63x objectives (1.4 NA).
477 Images were taken with a pixel size of 90 nm. Widefield imaging was performed in a Zeiss AxioVision
478 fluorescence microscope and a 3DHISTECH panoramic MIDI II digital slide scanner. Settings were
479 adjusted for each individual experiment and kept for all conditions. Image processing was performed
480 using FIJI (Schindelin et al., 2012), CaseViewer and Hyugens (SVI, The Netherlands) software. For
481 Thbs4, Nestin and DCX immunofluorescence, wholemount SVZ preparations and AAV-HAS2
482 analysis, tile-scan was done using the navigator plug-in in the LasX software. For Nestin
483 immunofluorescence, images were deconvoluted using the Hyugens software.

484 SVZ images were reconstructed using the tile-scan tool (LasX software, Leica). SVZ region of interest
485 (ROI) was delimited manually and expression of each marker was measured in the SVZ ROI.
486 Quantification of all markers was done manually, except for HABP quantification, which was done
487 with custom FIJI scripts for in vitro (https://github.com/MariArdaya/Hyaluronan_v2) and in vivo
488 analysis (https://github.com/SoriaFN/Tools). The HABP signal was segmented with an automatic
489 threshold and colocalized with other markers such as CD44. This, combined with cell segmentation,
490 based on the Thbs4 mask, allowed for quantification of HABP area and number of spots inside cells,
491 outside cells or at the membrane (with a band ROI of 1 μm). The Analyze Skeleton and Fractal count
492 plugins were used on the skeletonized segmented HABP to quantify the longest shortest path and the
493 fractal dimension, respectively, to reveal the length and interconnectivity of the matrix network (Casey
494 et al., 2024; Soria et al., 2020).

495

496 **Western blot**

497 The SVZ was freshly dissected on ice and minced using a micro grinder. Total protein was extracted
498 after tissue lysis with RIPA buffer (Thermo Fisher Scientific #89900) and Protease and Phosphatase
499 Inhibitor Cocktail 100X (Thermo Fisher Scientific). Samples were lysed in ice for 10 minutes and
500 sonicated using an Ultrasonic Processor (Hielscher Ultrasound Technology). Sonicated tissues were
501 centrifuged at 1200 rpm for 10 min and 4°C to remove insoluble fragments. Protein concentration was

502 measured by chemioluminescence using RC DC™ Protein Assay (Bio-Rad). Forty micrograms of
503 protein samples were denatured in reducing loading buffer containing β-mercaptoethanol (M3148,
504 Sigma) and 0.0002% bromophenol blue at 95°C for 5 minutes and separated in pre-casted 12% or 7.5%
505 Tris-Glycine polyacrylamide gel using the Criterion cell system (Biorad). Electrophoresis was
506 conducted at 80 V in a Tris-Glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS in dH2O, pH 8.3).
507 After complete separation, proteins were transferred to a nitrocellulose membrane (Biorad) using Trans-
508 Blot® TurboTM Transfer System (Bio-Rad). Nitrocellulose membranes were incubated in blocking
509 solution (Tris-Buffer Solution (TBS), 0.1% Tween-20 and 5% BSA) for one hour at room temperature.
510 Primary antibodies were incubated overnight at 4°C. Horseradish peroxidase-conjugated secondary
511 antibodies were incubated in blocking buffer for one hour at room temperature. Immunodetection was
512 performed by electrochemical luminescence in a Chemidoc MP (Biorad) and the intensity of the bands
513 was quantified using ImageLab (Bio-Rad) software corrected by Gaussian curves.

514

515 **qRT-PCR**

516 Fresh SVZ tissue from ipsilateral hemisphere was isolated as previously described from sham, 15 and
517 30 days after MCAO animals to analyze the expression of several genes (Supplementary Table 2). After
518 mechanical trituration, RNA was extracted from the tissue after mechanical trituration following the
519 manufacturer´s protocol (NZYTECH Total RNA Isolation kit protocol (NZYTECH, MB13402). RNA
520 concentration was measured by spectrophotometry using the Nanodrop 2000c spectrophotometer
521 (Thermo-Scientific, USA). Real time polymerase chain reaction (RT-PCR) from 10 ng of RNA was
522 performed using the One-step NZYTECH RT-qPCR Green kit according to manufacturer´s protocol
523 (NZYTECH, MB34302). RT-PCR was normalized using the housekeeping genes hypoxanthine
524 phosphoribosyl-transferase 1 (HPRT1, Qiagen QuantiTect Primer Assay, Barcelona, Spain). Specificity
525 of each amplification was confirmed by melting curve analysis. PCR reactions were performed in a
526 CFX96 Detection System (BioRad, Madrid, Spain). Semi-quantification was performed using the 2-

527 ΔΔCt algorithm. Unsupervised hierarchical distance matrix and distance matrix map was developed
528 using Orange3 software (Python v3.0).

529

530 **Statistical analysis**

531 Statistical analysis was done using GraphPad Prism software (version 8.0; GraphPad software). Data
532 are presented as mean ± SEM unless specified otherwise. Statistical analysis was performed using
533 unpaired Student´s t-test for independent conditions and paired Student´s t-test for dependent
534 conditions. In addition, one-way and two-way ANOVA were also performed to compare more than 2
535 groups among them. Tukey and Dunnett post-hoc tests were used. For HABP skeleton analysis,
536 Kruskal-Wallis analysis was performed to compare cumulative frequency curves in more than 2 groups.
537 *P* value was considered significant at $p < 0.05$. Sample number (*n*), *p* values and statistical test are
538 indicated in figure legends.

539

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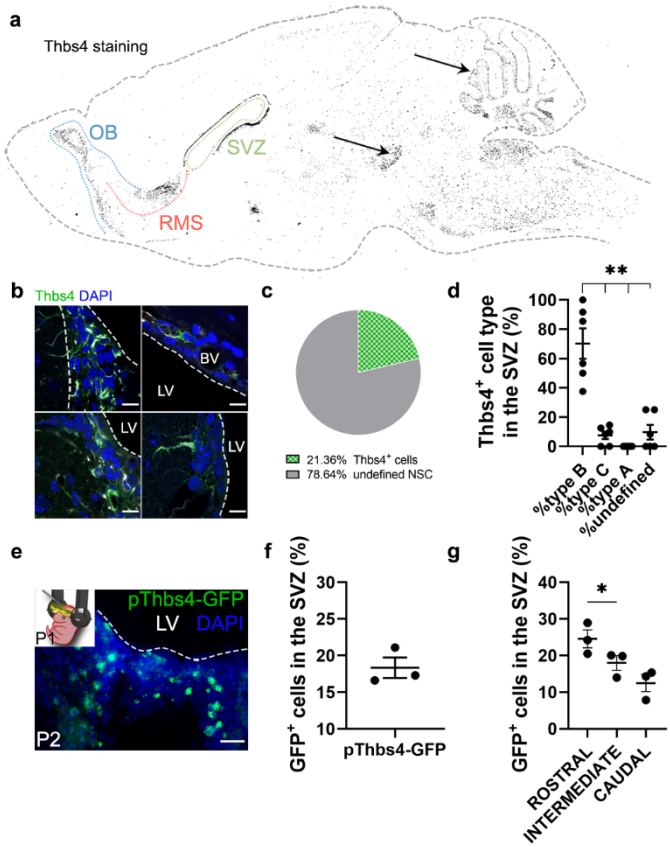
718

719 **FIGURES**



720

721 **Figure 1 – Thbs4 labels type-B cells in the SVZ neurogenic niche**. (a) Schematic drawing of the brain showing Thbs4
722 labeling throughout the mouse brain. Thbs4 was primarily found in the SVZ and RMS of the adult mouse telencephalon.
723 Arrows indicate non-specific Thbs4 staining in cerebellum and ventral tegmental area. (b) Thbs4 positive cells in the SVZ
724 displayed various morphologies. (c) Proportion of Thbs4-positive cells relative to the total NSCs population in the SVZ. (d)
725 Classification of Thbs4-positive cells in the SVZ based to their NSC morphology. (e) Electroporation of pThbs4-GFP at P1
726 revealed Thbs4 expression in the postnatal dorsal SVZ. (f) Quantification of Thbs4-reporter expression showing the proportion
727 of NSC expressing Thbs4 postnatally in the dorsal SVZ. (g) Thbs4-GFP reporter expression was upregulated in the rostral
728 SVZ. BV: Blood vessel; LV: Lateral ventricle. Scale bar = 10 µm (b) and 50 µm (e). Bars represent mean ± SEM. *$p < 0.05$
729 and **$p < 0.01$, Tukey post hoc test (after one-way ANOVA was significant at $p < 0.05$).

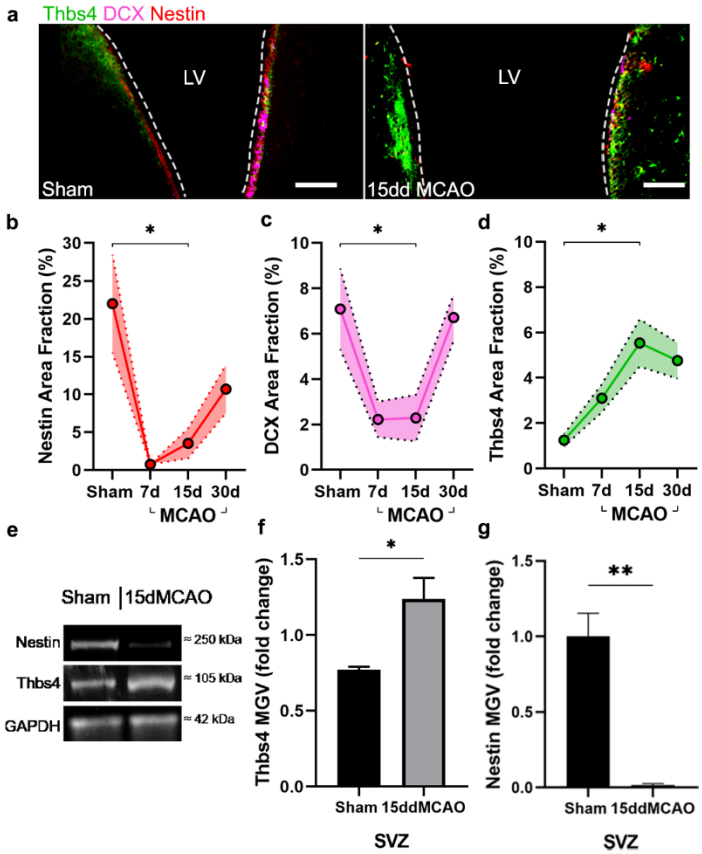
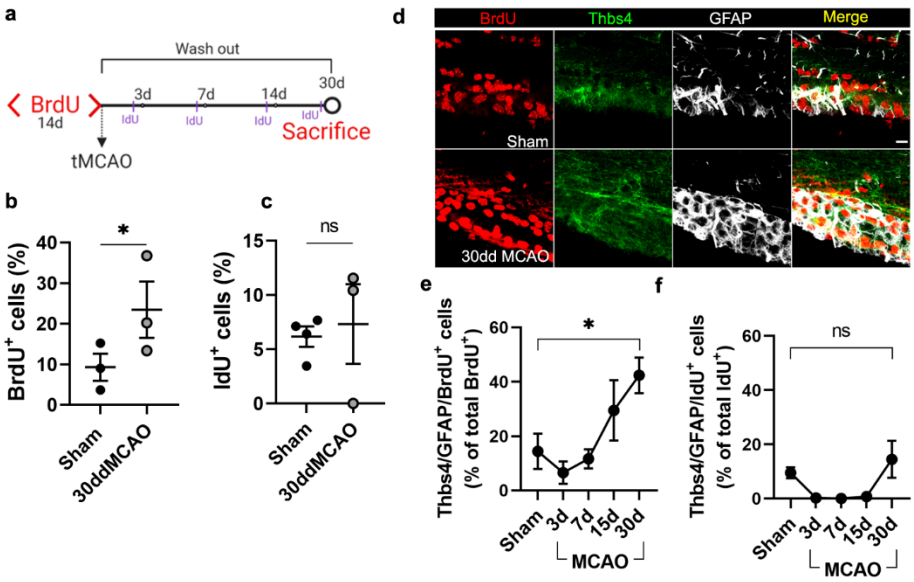


Figure 2 – Thbs4 expression is upregulated in the SVZ after brain ischemia. (a) Representative images of Thbs4 (green), Nestin (red) and DCX (magenta) in the SVZ under physiological conditions (left) and 15 days post-injury (dpi) (right). (b, c) The number of actively proliferating NSCs and neuroblast decreases at 7 and 15 dpi in the SVZ, as shown by reduced Nestin (b) and DCX (c) levels, respectively. (d) Thbs4 expression increases in the SVZ over time following ischemic injury. (e) Representative western blot images of Nestin and Thbs4 in sham and 15 dpi SVZ. (f, g) Quantification of mean gray value (MGV) from (e), normalized to GAPDH, shows an increased in Thbs4 (f) and a decrease in Nestin (g) in the 15 dpi SVZ. Scale bar = 100 µm in (a). n = 6 (b, c, d) and 3 (e, f, g) per condition. Bars represent mean ± SEM. *$p < 0.05$ and **$p < 0.01$; two-tail Student´s t test (sham vs. 15ddMCAO) and Tukey post-hoc test (after one-way ANOVA was significant at $p < 0.05$). See also Supplementary Figures 1 and 2.



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741

742 **Figure 3 – Thbs4-positive cells derive from B-type NSCs in the SVZ after brain ischemia.** (a) Experimental design:
743 chronic BrdU (1% in water) treatment was administered for two weeks. BrdU labeling persisted only in B-type NSCs 30 days
744 after treatment. Animals were injected with IdU (50 mg/kg) three times the day before euthanasia to label proliferating cells.
745 (b, c) BrdU (b) and IdU (c) positive cells in the SVZ. Only BrdU-positive cells showed a significant increase at 30 dpi. (d)
746 Representative confocal images showing Thbs4, GFAP and BrdU levels in sham (top) and 30 dpi mice (bottom). (e, f)
747 Thbs4/GFAP/BrdU positive cells (slow proliferative cells) increased in the SVZ at 30 dpi (e), while no changes were observed
748 in Thbs4/GFAP/IdU positive cells (fast proliferative cells). Scale bar = 10 µm (d). n = 4 (sham) and 3 (MCAO). Bars represent
749 mean ± SEM. *$p < 0.05$ by two-tail Student´s t-test (sham vs. 30dd MCAO). See also Supplementary Figure 3.

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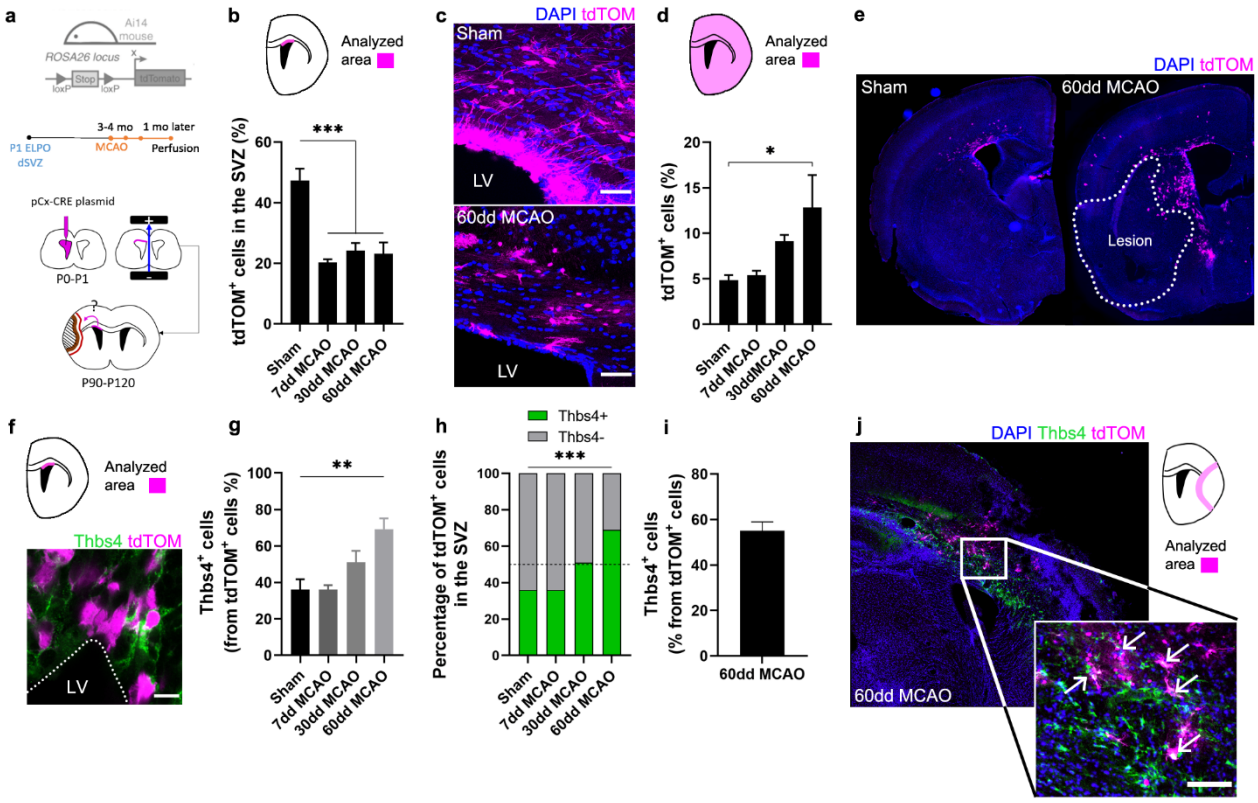

751

752 **Figure 4 – Ischemia-induced Thbs4 astrocytes migrate from the SVZ to ischemic areas.** (a) Experimental design: Ai14,
753 ROSA26-CAG-tdTomato transgenic mice were electroporated in the dorsal SVZ at P1. The pCAGGSx-CRE plasmid induced
754 tdTomato (tdTOM) expression in dorsal NSCs. MCAO was performed 3-4 months later in the same electroporated hemisphere.
755 Mice were analyzed at 7, 30, and 60 dpi. (b) Quantification of tdTOM-positive cells shows a decrease in the dorsal SVZ
756 following brain ischemia. (c) Representative images of tdTOM-positive cells in sham (top) and 60 dpi SVZ (bottom). (d)
757 Quantification of tdTOM-positive cells in the whole brain shows a gradual increase outside the SVZ after brain ischemia. (e)
758 Representative images of tdTOM expression in sham (left) and 60 dpi mice (right). (f) Representative image of Thbs4/tdTOM-
759 positive cells in the dorsal SVZ. (g) Quantification shows an increase in Thbs4/tdTOM-positive cells in the SVZ at 30 and 60
760 dpi. (h) The proportion of Thbs4-positive cells within the tdTOM-positive pool increases in the SVZ at 30 and 60 dpi. (i)
761 Around 50% of tdTOM-positive cells expressed Thbs4 in the infarcted tissue at 60 dpi. (j) Representative image of Thbs4 and
762 tdTOM expression near the damaged area. Scale bar = 50 µm (c), 10 µm (f), and 100 µm (g). n = 6 animals per condition. Bars
763 represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by Chi-square test (h) and Tukey post-hoc test (after one-
764 way ANOVA was significant at $p < 0.05$). See also Supplementary Figure 4.

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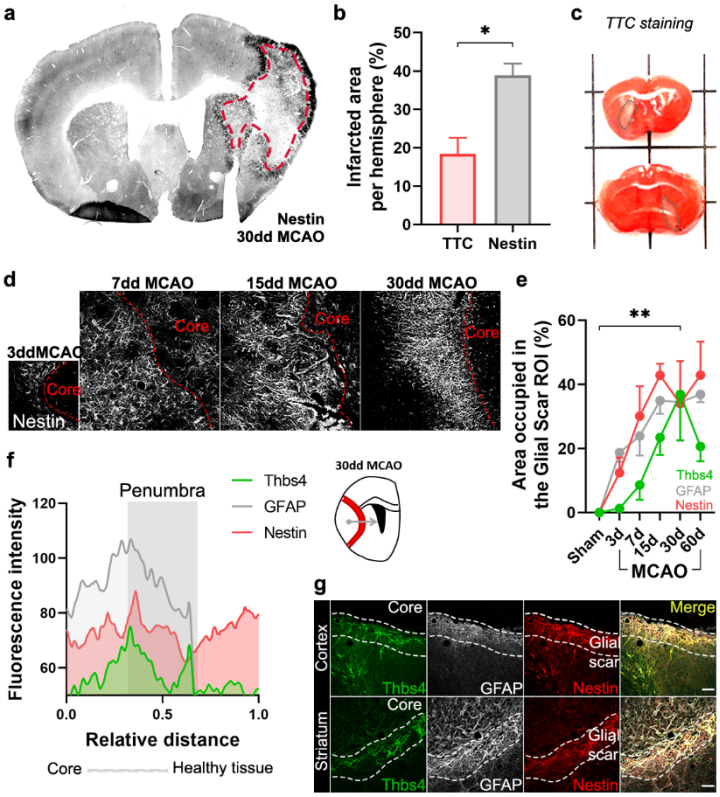


Figure 5 – Ischemia-induced Thbs4 astrocytes accumulate at the glial scar. (a) The glial scar could be observed at 30 dpi using Nestin immunofluorescence. (b) Classical TTC technique underestimated ischemic regions compared with Nestin immunofluorescence. (c) Representative images of TTC staining in infarcted brains. (d) Representative confocal images showing the time course of Nestin expression at 3, 7, 15 and 30 dpi. (e) The Nestin-positive glial scar was established by 15 dpi, while Thbs4 astrocytes arrived at the glial scar later, by 30 dpi. (f) Fluorescence profile of Thbs4, GFAP and Nestin marker from the core to penumbra areas shown Thbs4 was mainly located at the borders of the Nestin-positive glial scar. (g) Representative images of Thbs4 (green), GFAP (white) and Nestin (red) markers in sham (top) and 30 dpi mice (bottom). Scale bar = 50 µm (g). n = 3 animals per condition. Bars/lines represent mean ± SEM. *$p < 0.05$ and **$p < 0.01$ by two-tail Student´s t test (TTC vs. Nestin) and by Dunnett posthoc test (after two-way ANOVA was significant at $p < 0.05$). See also Supplementary Figure 5.

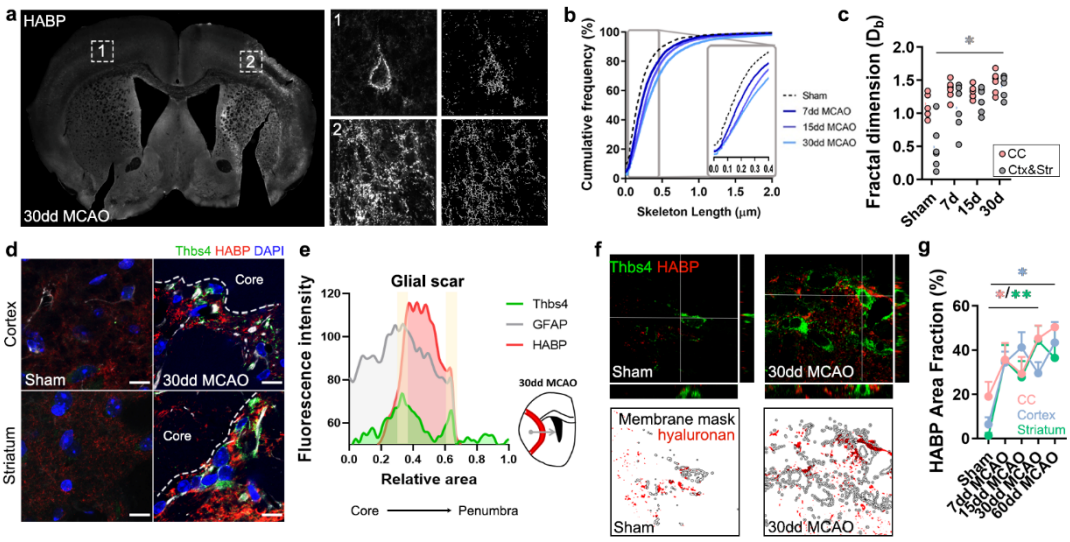


Figure 6 – Thbs4-positive astrocytes produce hyaluronan at the glial scar after MCAO. (a) Representative images of hyaluronic acid (HA) labeled with HABP in the contralateral (1) and ipsilateral hemisphere (2). Only perineuronal nets are clearly visible in the healthy tissue, while a dense interstitial matrix is observed in ischemic areas. (b) Cumulative distribution of skeletonized HA signal in sham and infarcted regions of 7, 15 and 30 dpi mice ($p < 0.0001$). (c) Quantification of fractal dimension showed increased extracellular matrix interconnectivity in the infarcted cortex and striatum (Ctx & Str) at 30 dpi. (d) Representative confocal images of Thbs4 (green) and HABP (red) in the cortex and striatum of sham and 30 dpi mice. Note the HA labelling on the surface of Thbs4-positive cells. (e) Fluorescence intensity profile of Thbs4, GFAP and HABP markers from the lesion core to penumbra areas. Thbs4 expression was mainly restricted to the borders of the HA-positive glial scar at 30 dpi. (f) Representative confocal images of Thbs4 and HABP markers in sham and 30 dpi mice (top). Examples of HA (red) occupying the membrane mask of Thbs4-positive astrocytes in sham and 30 dpi mice (bottom), showing increased HA synthesis after MCAO. (g) Quantification of membrane-bound HA showed increased HA along Thbs4-positive membrane after brain ischemia (corpus callosum: pink; infarcted cortex: blue; infarcted striatum: green). Scale bar = 10 µm (e). n = 6 animals per condition. *$p < 0.05$ and ****$p < 0.0001$ by Krustal Wallis test and two-way ANOVA (significant at $p < 0.05$). See also Supplementary Figures 6 and 7.

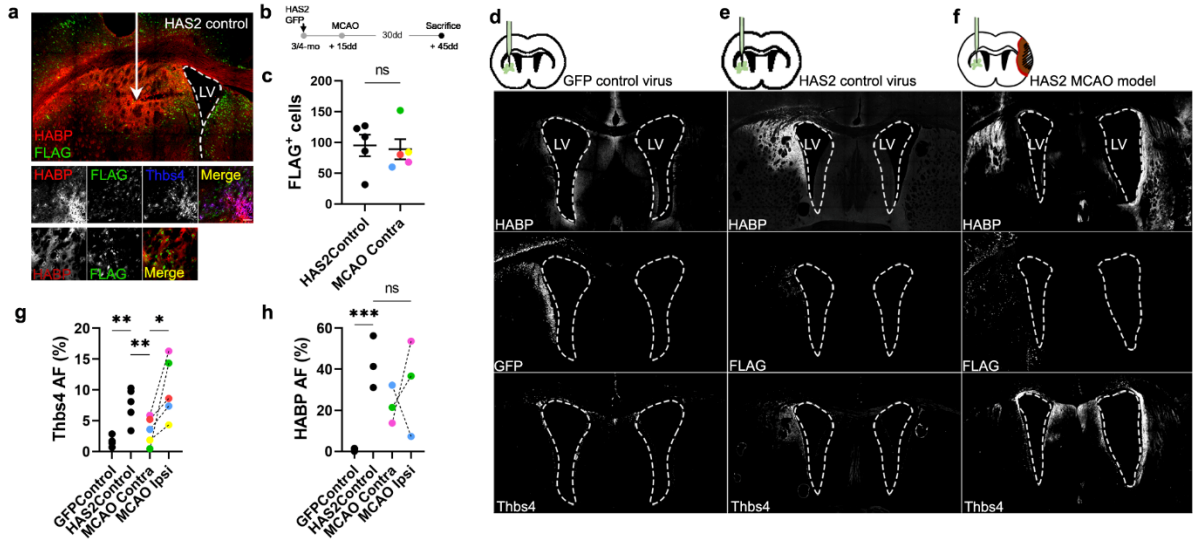


Figure 7 – Hyaluronan accumulation is sufficient but not exclusive to recruit Thbs4 astrocytes. (a) Representative images of HA accumulation caused by viral-mediated overexpression of HAS2 from naked mole rat. (b) Experimental design: HAS2 viral injections were performed 2 weeks before MCAO. Animals were sacrificed 30 days after MCAO. We used standard GFP virus as control. Two groups of HAS2-injected mice were studied, with only one subjected to MCAO. (c) FLAG-positive cells (HAS2 virus tag) did not show changes in either HAS2 control (HAS2Control) or HAS2-MCAO group (contralateral hemisphere). (d, e, f) Representative images of HABP, GFP and Thbs4 markers in GFP control virus (d), HAS2 control virus (e) and HAS2 MCAO group (f). (g) Thbs4 expression increased after HAS2 viral-mediated overexpression. However, Thbs4 was upregulated in the ischemic hemisphere when MCAO was performed together with the HAS2 viral infection (MCAO ipsi). GFP control virus did not induce increase of Thbs4. (h) HABP was used as a control for HA accumulation. Thbs4 astrocytes in HAS2 MCAO group increased in the ischemic hemisphere even though HA accumulation in both hemispheres (HAS2 and MCAO hemisphere), suggesting additional ischemia-induced signals recruit Thbs4 astrocytes to infarcted areas. Scale bar = 100 µm (a). n = 6 animals per condition. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by two-tail Student´s t-test (HAS2Control vs. MCAO Contra) and Tukey posthoc test (after one-way ANOVA was significant at $p < 0.05$).

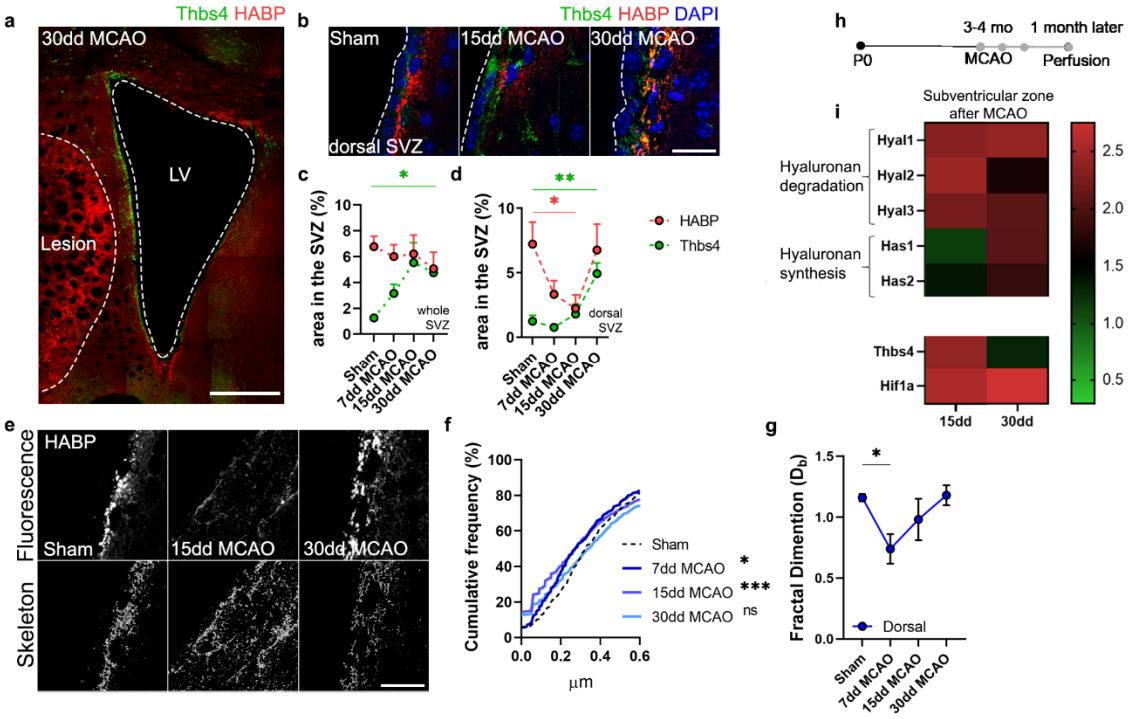
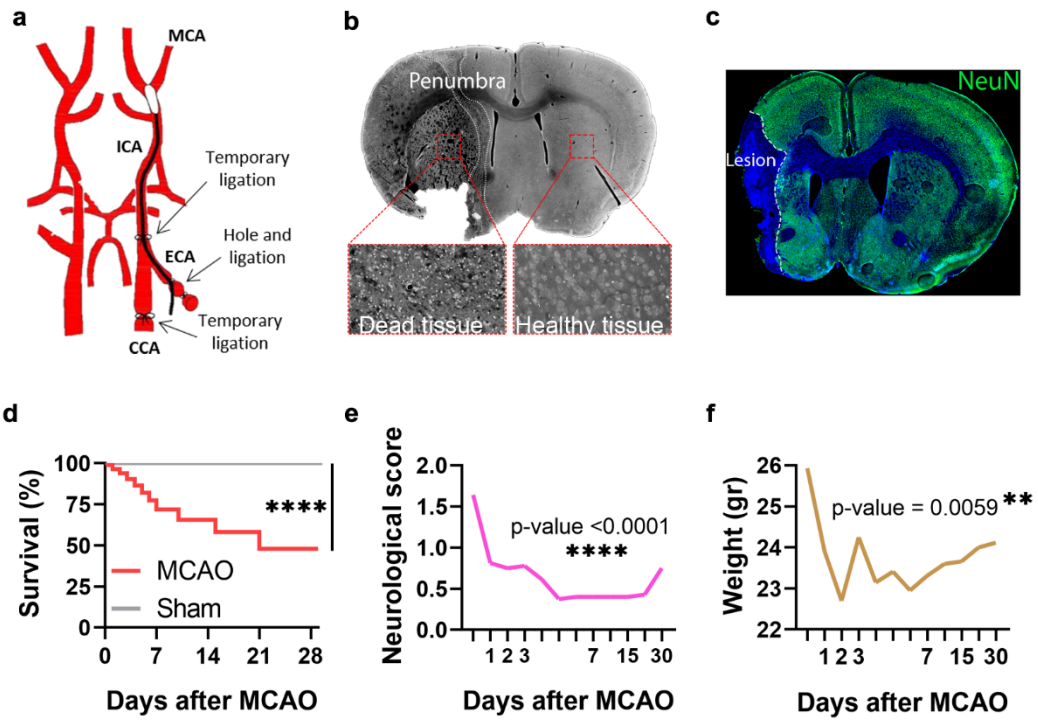
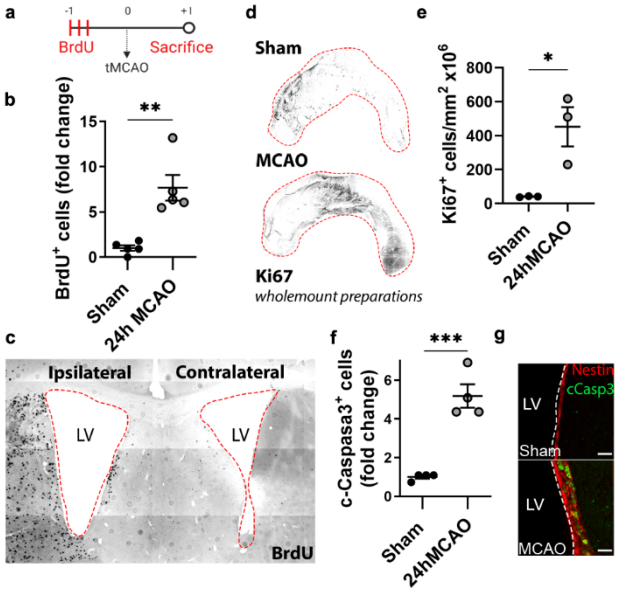


Figure 8 – HA degradation in the SVZ correlates with Thbs4 response to brain ischemia. (a) Representative image of Thbs4 and HA (HABP) markers in the SVZ at 30 dpi. (b) Representative images of Thbs4 and HA in the sham and 15, 30 dpi SVZ. (c, d) HA levels did not change in the entire SVZ after MCAO (c) but decreased significantly in the dorsal SVZ (d). Thbs4 expression increased throughout the SVZ after MCAO (c), with a marked rise at 30 dpi in the dorsal SVZ (d), coinciding with HABP reduction. (e) Representative confocal (top) and skeletonized (bottom) images of HABP in the dorsal SVZ of sham, 15 and 30 dpi mice. (f) Cumulative distribution of HA skeletons showed a reduction in the length of HA cable-like structures only in the dorsal SVZ at 7 and 15 dpi. (g) The interconnectivity of the extracellular matrix decreased by 7 dpi in the dorsal SVZ, as measured by fractal dimension. (h) Experimental design for qPCR: 3–4-month-old mice were subjected to MCAO, and fresh SVZ tissue was extracted 15 and 30 days after MCAO for RT-qPCR analysis. (i) Hyaluronan degradation genes (Hyal1, Hyal2 and Hyal3) increased by 15 and 30 dpi in the SVZ, while hyaluronan synthase genes (HAS1 and HAS2) were overexpressed later, at 30 dpi. Thbs4 and Hif1a were also upregulated by 15 and 30 dpi in the SVZ. Scale bar = 100 µm (a). n = 6 (c, d, e and g) and 3 (i). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Krustal Wallis test and Tukey post-hoc test (after one-way ANOVA was significant at $p < 0.05$).

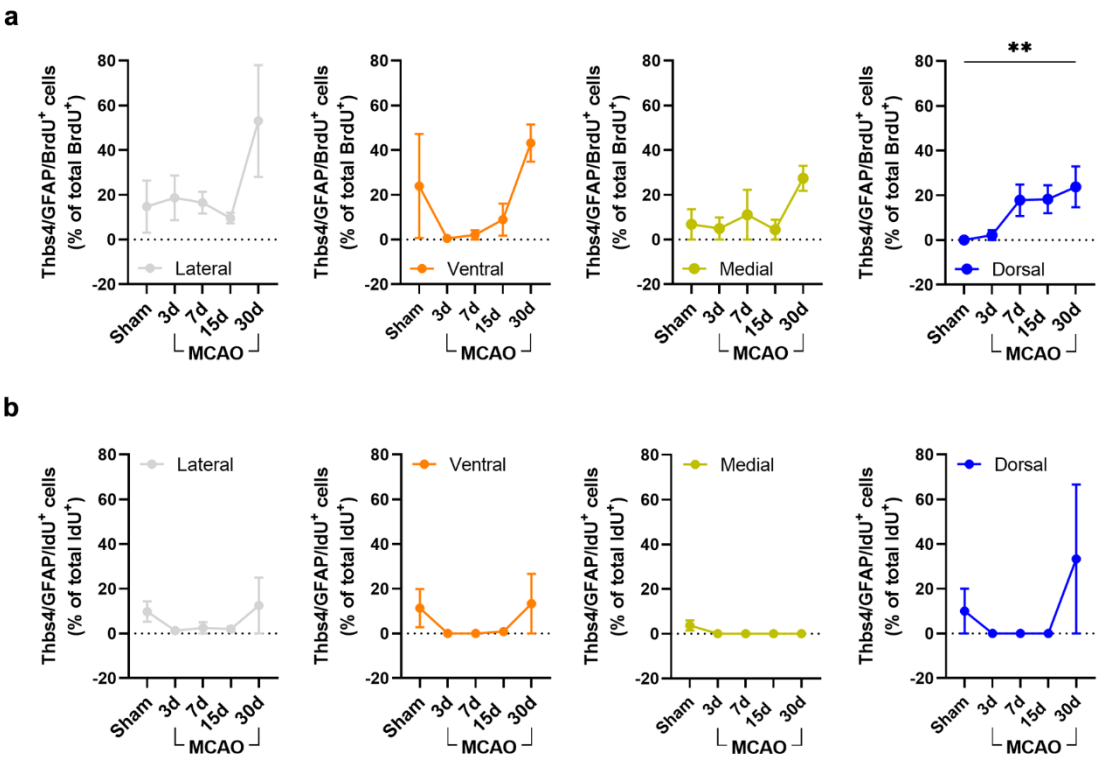
SUPPLEMENTARY INFORMATION



Supplementary Figure 1 – Characterization of the Middle Cerebral Artery Occlusion (MCAO) mouse model of brain ischemia. Related to Figure 2. (a) Scheme of the procedure: 10 mm silicon-coated filament was inserted through an external artery and re-directed to the internal one. Ischemia was induced after 60 minutes of occlusion. After that, the filament was removed, and animals were observed every day before euthanasia. (b) Volume of infarction was observed by cresyl violet staining. (c) Neuronal death was observed by NeuN immunofluorescence. (d) Around 50% of ischemic mice survived at 28 dpi. (e) Ischemic animals ameliorated neurological symptoms over time. (f) Weight was measured as controls for mice healthcare. n = 17 (d, e, f). $^{**}p < 0.01$ and $^{****}p < <0.0001$ by survival curve test and Tukey post-hoc test (after one-way ANOVA was significant at $p < 0.05$).



Supplementary Figure 2 – Cell proliferation in the SVZ after brain ischemia. Related to Figure 2. (a) Experimental design: three BrdU (50mg/kg) injections were performed the day before MCAO protocol. Animals were sacrificed 24 hours after the brain stroke model. (b) BrdU-positive cells increased in the SVZ 24 hours after MCAO. (c) Representative image of BrdU positive cells in the ipsilateral (left) and contralateral (right) hemisphere 24 hours after MCAO. (d) Ki67 marker was also used to analyze cell proliferation in wholemount preparations of the SVZ. (e) Ki67 positive cells increased in the SVZ 24 hours after MCAO. (f) The Cleaved-Caspase 3 marker increased in the SVZ 24 hours after MCAO. (g) Cleaved-caspase 3 marker was used to assess apoptosis in the SVZ after brain ischemia. Scale bar = 15 µm (g). n = 5 (sham) and 4 (MCAO). Bars represent mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by two-tail Student´s t-test (sham vs. 24h MCAO).



845

846 **Supplementary Figure 3 – Thbs4 astrocytes derive from slow proliferative cells after brain ischemia.** Related to Figure
847 3. (a) Slow proliferative cells were quantified in the lateral (grey), ventral (orange), medial (yellow) and dorsal (blue) SVZ.
848 We only observed an increase of Thbs4/GFAP/BrdU positive cells in the dorsal SVZ 30 dpi. (b) Thbs4/GFAP/IdU positive
849 cells did not show changes in the lateral (grey), ventral (orange), medial (yellow) and dorsal (blue) SVZ after brain ischemia.
850 n = 4 (sham) and 3 (MCAO). $^{**}p < 0.01$ by by two-tail Student´s t-test (sham vs. 30dd MCAO).

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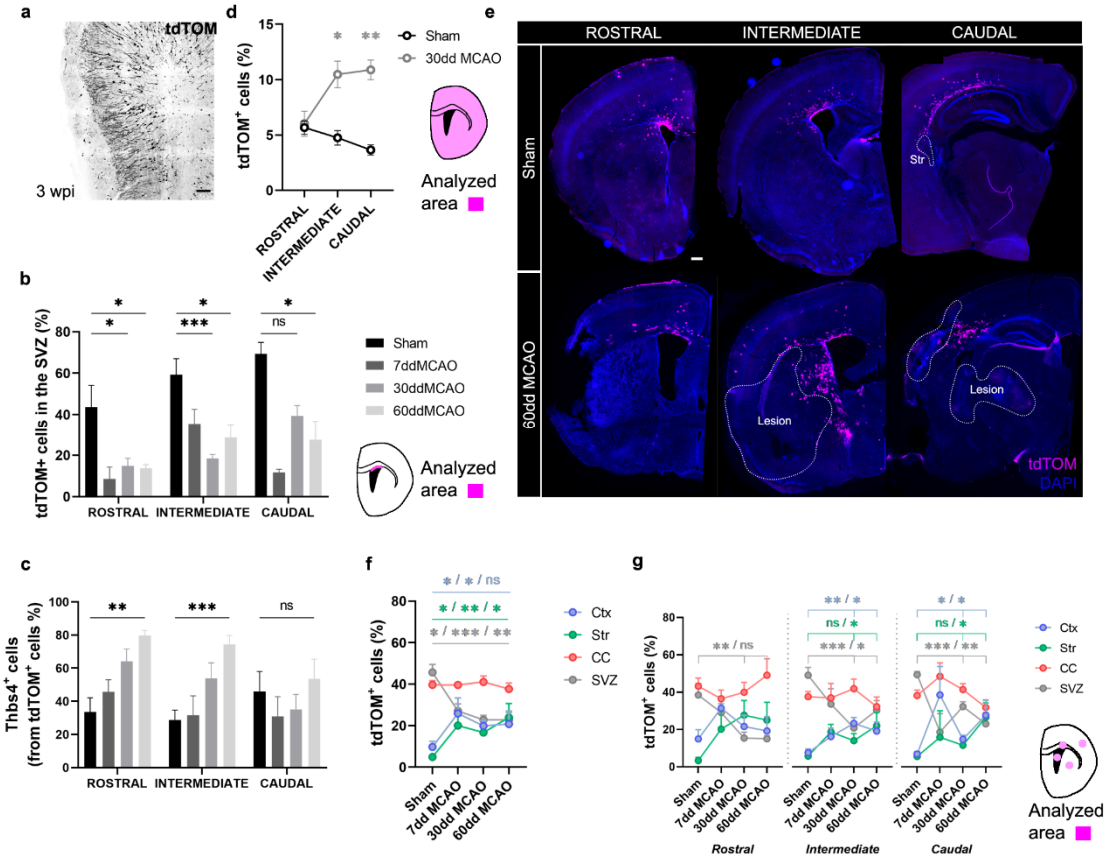
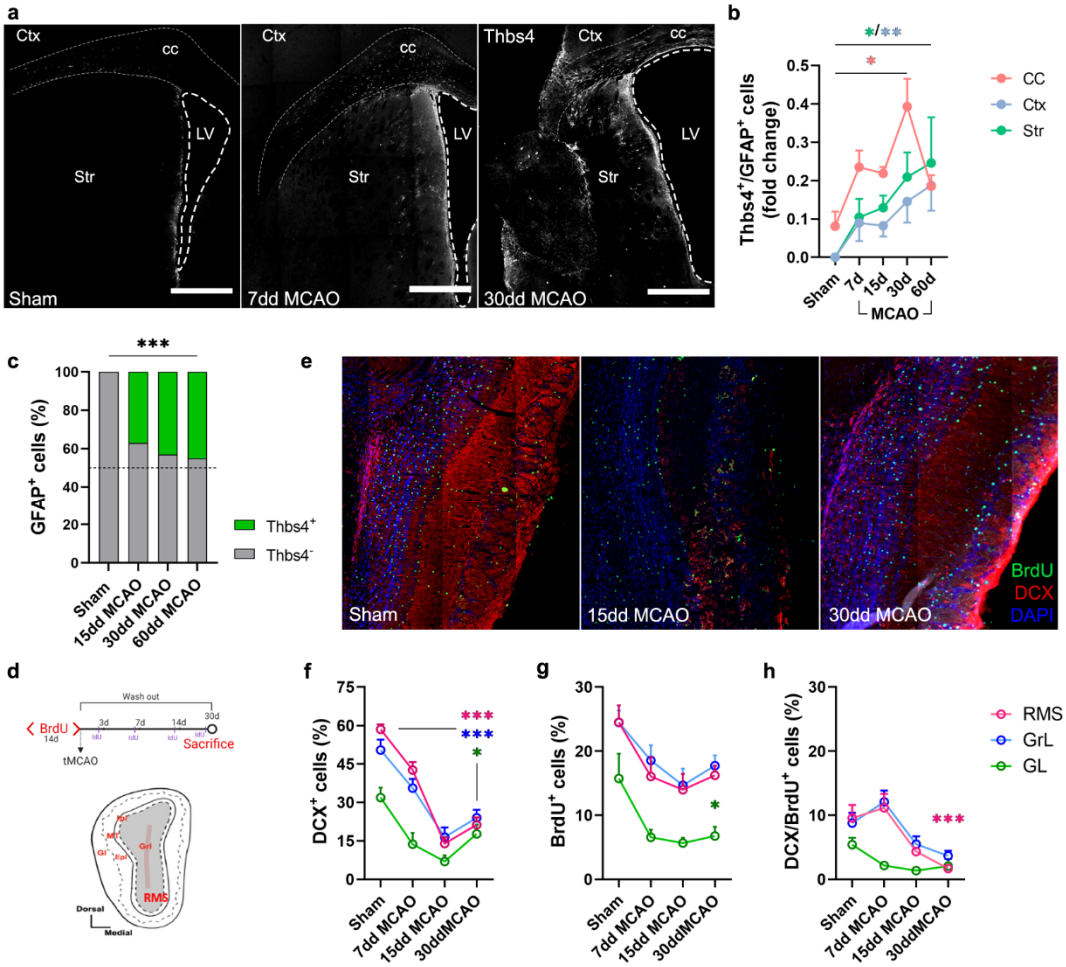
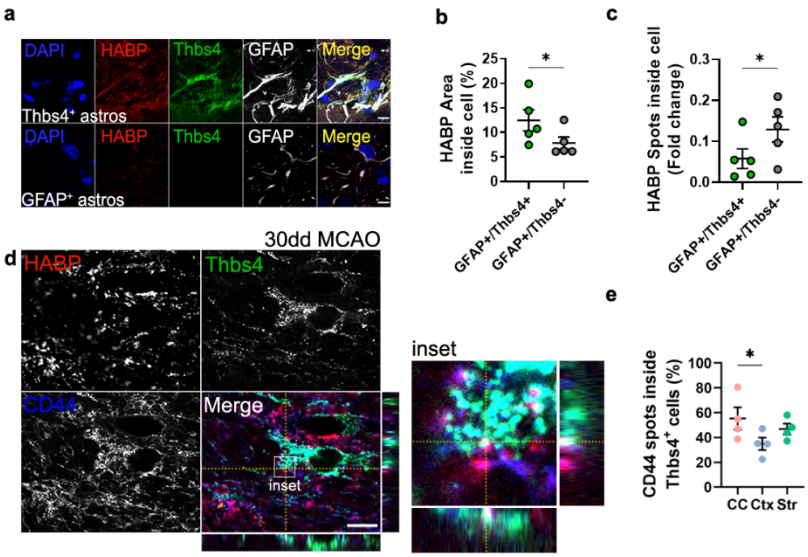


Supplementary Figure 4 – Ischemic-induced Thbs4 astrocytes migrate preferentially to caudal infarcted areas. Related to Figure 4. (a) Representative image of tdTOM positive cells in the OB three weeks after electroporation (3wpi). (b) tdTOM-positive cells decreased in the rostral, intermediate and caudal SVZ 7, 30 and 60 dpi. (c) Thbs4/tdTOM positive cells increased in rostral and intermediate SVZ 30 and 60 dpi. (d) Expression of tdTomato increased in intermediate and caudal brain areas at 30 dpi. (e) Representative images of tdTOM expression in rostral (left), intermediate (middle) and caudal (right) brain areas of sham (top) and 60 dpi mice (bottom). (f) tdTOM positive cells increased in the infarcted cortex (Ctx) and striatum (Str) whereas decreased in the dorsal SVZ (SVZ). No changes were observed in the corpus callosum (CC). (g) tdTOM positive cells only increased in the infarcted cortex and striatum at intermediate and caudal brain regions. Scale bar = 100 µm (a) and 1000 µm (e). n = 6 animals per condition. Bars represent mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Dunnett/Tukey post-hoc test (after two-way ANOVA was significant at $p < 0.05$).



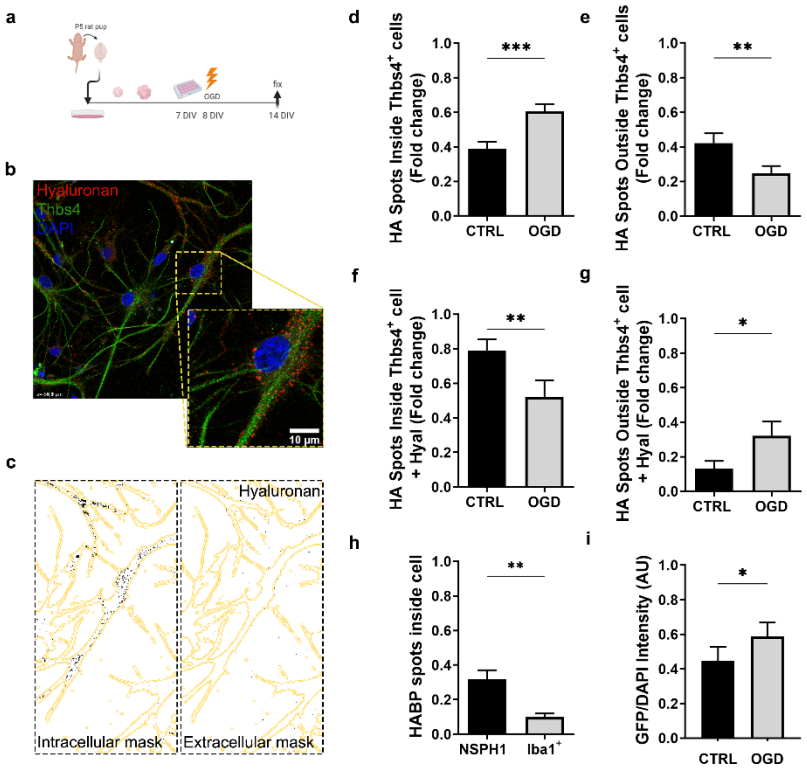
Supplementary Figure 5 – SVZ NSC stop producing newborn neurons to send newborn astrocytes to the ischemic areas. Related to Figure 5. (a) Thbs4 expression in the corpus callosum (cc), infarcted cortex (Ctx) and infarcted striatum (Str) of sham and 7 and 30 dpi mice. (b) Thbs4 increased by 30 dpi in the corpus callosum (cc) and 60 dpi in the infarcted cortex (Ctx) and infarcted striatum (Str). (c) Expression of Thbs4 increased in total GFAP population after 15, 30 and 60 dpi. (d) Experimental design: chronic BrdU (1% in water) treatment was administered for two weeks. BrdU only persisted in slow proliferative NSC (Codega et al., 2014). DCX and BrdU positive cells were measured in the OB layers 7, 15 and 30 dpi. (e) Representative images of BrdU and DCX marker in sham (left), 15 (middle) and 30 (right) dpi mice OB. (f) DCX positive cells decreased 15 and 30 dpi in the RMS, granular OB layer (GrL) and glomerular OB layer (GL). (g) BrdU positive cells only decreased 30 dpi in the glomerular OB layer (GL). Double DCX/BrdU positive cells significative decreased in the RMS 30 dpi. In (f) and (g), values are normalized to total DAPI nuclei (100%). Scale bar = 500 µm (a). n = 6 animals per condition. Lines represent mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Chi-square test and Tukey post-hoc test (after two-way ANOVA was significant at $p < 0.05$).



875

876 **Supplementary Figure 6 – Thbs4 astrocytes internalize HA in the infarcted areas.** Related to Figure 6. (a) Representative
877 images of Thbs4/GFAP ("newborn astrocytes", top) and GFAP-only ("local astrocytes", bottom) cells in the infarcted areas at
878 30 dpi. (b) Thbs4-positive astrocytes internalized more HA compared to local astrocytes only labelled with GFAP marker. (c)
879 However, the number of HA spots internalized was higher in local astrocytes compared with Thbs4 astrocytes, suggesting that
880 Thbs4 internalize HA in fewer vesicles. (d) Representative images of HABP (red), Thbs4 (green) and CD44 (blue) at the lesion
881 border. Inset shows an orthogonal projection of Thbs4/CD44/HABP-positive intracellular vesicle. (e) Thbs4 positive
882 astrocytes expressed a high rate of CD44 receptors so much in corpus callosum (CC), infarcted cortex (Ctx) as in infarcted
883 striatum (Str). Scale bar = 10 µm (c, f). n = 5 animals per condition. Bars represent mean ± SEM. *$p < 0.05$ and **$p < 0.01$ by
884 Tukey posthoc test (after two-way ANOVA was significant at $p < 0.05$) and two-tail Student´s t-test (Thbs4 vs GFAP).

885



886

887 **Supplementary Figure 7 – In vitro NSC-derived Thbs4 cells synthetize HA after a hypoxic-ischemic condition.** Related
888 to Figure 6. (a) Experimental design: P4 pup rats were sacrificed, and SVZ NSC were extracted and cultured in the proliferative
889 medium for one week. After that, neurospheres were disaggregated and seeded isolated in a culture dish. The following day,
890 oxygen and glucose deprivation protocol (OGD) was performed. Cells were fixed 7 days after culture them. (b) Representative
891 images of in vitro Thbs4 positive cells (green) and HA (red). (c) HA spots were measured inside and outside Thbs4 positive
892 cells. (d) HA spots increased inside Thbs4 positive astrocytes after OGD whereas (e) decreased in the extracellular space.
893 However, when NSC were exposed to hyaluronidases in order to remove extracellular HA, Thbs4 positive cells decreased
894 internalization of HA spots (f) and increased extracellular HA spots (g). (h) Neurosphere-derived NSC (NSPH1) internalized
895 more HA spots compared with Iba1-positive cells. (i) Metalloproteinase activity was higher in OGD NSC cultures compared
896 to control condition. Scale bar = 10 µm (b). n = 15 samples per condition. Bars represent mean ± SEM. *$p < 0.05$, **$p < 0.01$
897 and ***$p < 0.001$ by two-tail Student´s t-test (CTRL vs. OGD; NSPH1 vs. Iba1^{+}).

898

899

Table 1. Primary antibodies used for in vivo immunofluorescence protocol

Antibody	Host	Isotope	Dilution	Reference	Company
Thbs4	Mouse	Monoclonal IgM kappa light chain	1/400	Sc-390734	Santa Cruz Biotechnology, USA.
Thbs4	Goat	Polyclonal IgG	1/200	AF2390	R&B systems, USA
S100β	Rabbit	Polyclonal	1/400	Z0311	Dako, Agilent Technologies, Inc.
S100β	Mouse	Monoclonal IgG2a	1/1000	MA5-12969	Invitrogen, Spain
GFAP	Mouse	Monoclonal IgG1	1/1000	MAB3402	Sigma, Spain
GFAP	Rabbit	Polyclonal	1/4000	Z0334	Dako, Agilent Technologies, Inc.
βIII tubulin	Rabbit	Polyclonal	1/400	ab18207	Abcam, UK
NeuN	Mouse	Monoclonal IgG1	1/1000	MAB377	Millipore, Germany
DCX	Rabbit	Polyclonal IgG	1/500	ab18723	Abcam, UK
Nestin	Chicken	Polyclonal IgY	1/1000	NES	Aves Lab, EEUU
Prominin 1	Rat	Monoclonal IgG1 kappa	1/300	14-1331-82	Invitrogen, Spain
EGFR	Mouse	Monoclonal	1/400	SAB4200809	Abcam, UK
BrdU	Rat	Monoclonal	1/400	MCA2060	Bio-Rad, USA.
BrdU	Mouse	Monoclonal IgG1, kappa	1/500	03-3900	Invitrogen, Spain
IdU	Mouse	Monoclonal IgG2b	1/100	MA5-24879	Invitrogen, Spain
Ki67	Rabbit	Polyclonal IgG	1/400	Ab15580	Abcam, UK
Cleaved-Caspase 3	Rabbit	Monoclonal IgG	1/300	#9664	Cell Signaling, Germany
CD44	Rat	Monoclonal IgG2b	1/500	MA517875	Invitrogen, Spain
FLAG	Mouse	Monoclonal IgG1	1/500	F1804	Sigma, Spain
Olig2	Mouse	Monoclonal IgG2aκ	1/1000	MABN50	Millipore, Germany

900

Table 2. Primer sequences for qPCR

901

Name	Sequence
Hyal1_FW	GTGCCAAGCCCTATGCTAATAAG
Hyal1_REV	GCATGTCCATTGCAAAGACTGA
Hyal2_FW	GTCCCACATACACCCGAGGA
Hyal2_REV	GGCACTCTCACCGATGGTAGA
Hyal3_Ffw	GGACGACCTGATGCAGACTATTG
Hyal3_REV	GGTCCCCCCAGAGTACCACT
Has1_FW	AGGGCTCTTAAAGGAGGAGTCC
Has1_REV	AGAAGGTAAACTGAGTCCCCAGAA
Has2_FW	CAAAGAGGTTCGTTCAAGTTCTGA
Has2_REV	TGTGTTTGTTTCCCACTAGCTCTC
Hif1a_FW	CATAAAGTCTGCAACATGGAAGGT
Hif1a_REV	ATTTGATGGGTGAGGAATGGGTT
HPRT1_FW	TGATAGATCCATTCCTATGACTGTAGA
HPRT1_REV	AAGACATTCTTTCCAGTTAAAGTTGAG

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