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SRF is required for maintenance of astrocytes in non-reactive state in the mammalian brain

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Mechanism regulating reactive astrogliosis

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78   **SRF is required for maintenance of astrocytes in non-reactive state in the mammalian**

79   **brain**

80

81   **ABSTRACT**

82   Astrocytes play several critical roles in the normal functioning of the mammalian brain including

83   ion homeostasis, synapse formation and synaptic plasticity. Following injury and infection or in

84   the setting of neurodegeneration, astrocytes become hypertrophic and reactive, a process termed

85   astrogliosis. Although acute reactive gliosis is beneficial in limiting further tissue damage,

86   chronic gliosis becomes detrimental for neuronal recovery and regeneration.   Several

87   extracellular factors have been identified that generate reactive astrocytes; however, very little is

88   known about the cell-autonomous transcriptional mechanisms that regulate the maintenance of

89   astrocytes in the normal non-reactive state. Herein, we show that conditional deletion of the

90   stimulus-dependent transcription factor, serum response factor (SRF) in astrocytes (Srf^{GFAP}-

91   CKO) results in astrogliosis marked by hypertrophic morphology and increased expression of

92   GFAP, vimentin and nestin. These reactive astrocytes were not restricted to any specific brain

93   region and were seen in both white and grey matter in the entire brain. This astrogliosis persisted

94   throughout adulthood concomitant with microglial activation. Importantly, the Srf mutant mouse

95   brain did not exhibit any cell death or blood brain barrier (BBB) deficits suggesting that

96   apoptosis and leaky BBB are not the causes for the reactive phenotype. The mutant astrocytes

97   expressed more A2 reactive astrocyte marker genes and the Srf^{GFAP}CKO mice exhibited normal

98   neuronal numbers indicating that SRF-deficient gliosis astrocytes are not neurotoxic. Together

99   our findings suggest that SRF plays a critical role in astrocytes to maintain them in a non-

100   reactive state.

101

SIGNIFICANCE STATEMENT

In response to CNS injury, infection and in neurodegeneration, astrocytes undergo complex structural and physiological changes termed as reactive gliosis. Currently, the molecular mechanisms that regulate the non-reactive state of the astrocytes are poorly understood. We report that the SRF transcription factor is required for the maintenance of astrocytes in the non-reactive state such that its conditional deletion in astrocytes results in widespread reactive astrogliosis. The SRF-deficient reactive astrocytes are persistent, non-proliferating and are not caused by cell death or impaired blood brain barrier integrity. In this regard, SRF regulates reactive astrocyte generation in the mammalian brain in a cell-autonomous manner.

INTRODUCTION

As an essential part of the central nervous system (CNS), astrocytes play critical roles in nearly every facet of its development and function including ion and neurotransmitter homeostasis, maintenance of the blood brain barrier (BBB), synapse formation and elimination, and synaptic transmission (Barres, 2008; Kimelberg, 2010; Kimelberg and Nedergaard, 2010). In addition, astrocytic dysfunction are central in several CNS disorders such as epilepsy, amyotrophic lateral sclerosis and Alzheimer's disease (Seifert et al., 2006; Phatnani and Maniatis, 2015). In response to CNS injuries and pathologies, astrocytes undergo a spectrum of gene expression as well as physiological and structural changes, a process known as reactive astrogliosis (Burda and Sofroniew, 2014; Liddelow and Barres, 2017). These astrocytic responses depend on the severity of the CNS trauma and can range from transient responses lasting a few days to a more permanent glial scar formation (Sofroniew, 2009, 2015).

4

124

125   Reactive astrogliosis is largely considered beneficial to the CNS, where reactive

126 astrocytes provide protection by several mechanisms, ranging from efficient uptake of

127 excitotoxic glutamate, preventing oxidative stress and reducing edema, to restricting

128 inflammation, facilitating blood brain barrier repair and restricting spread of infection (Hamby

129 2010; Escartin 2008; Pekny 2014). However, astrogliosis can also cause detrimental effects

130 wherein reactive astrocytes inhibit CNS regenerative responses, contribute to

131 neuroinflammation, generate reactive oxygen species and cause cell death (Pekny 2014;

132 Sofroniew 2014). Previous studies have identified several extracellular factors and intracellular

133 signaling pathways that regulate different aspects of astrogliosis including cytokines, growth

134 factors, purines, Endothelin-1, BMP receptors, Eph4 and Aquaporin 4 (Correa-Cerro and

135 Mandell, 2007; Kang and Hebert, 2011; Sofroniew, 2014).

136

137   Currently, we know little about the identities of transcription factors that are necessary to

138 maintain astrocytes in a non-reactive state. SRF is a stimulus-dependent transcription factor

139 important for several aspects of nervous system development (Knoll and Nordheim, 2009). SRF

140 has been shown to play a critical role in oligodendrocyte and astrocyte development (Stritt et al.,

141 2009; Lu and Ramanan, 2012) but its functions in astrocytes remain unknown. In this study, we

142 conditionally ablated SRF in astrocytes using a GFAP-Cre transgenic line (Bajenaru et al., 2002).

143 The brains of Srf^{GFAP}CKO mice exhibited reactive astrogliosis starting 3 weeks of age. These

144 reactive astrocytes were not restricted to any specific region and were seen throughout the brain.

145 The reactive gliosis persisted throughout adulthood with concomitant microglial activation. We

146 did not observe any changes in cell death or blood brain barrier integrity, indicating that these

147 extrinsic factors are unlikely the cause of gliosis. Together our findings suggest that SRF is a

148 critical cell autonomous regulator of non-reactive state of astrocytes throughout the brain.

149

150 **Materials and Methods**

151 **Animals.** *Srf*-floxed mice were obtained from Jackson laboratories (Stock No 006658). These

152 mice were bred with hGFAP-Cre (generously provided by Dr. David Gutmann, Washington

153 University School of Medicine, St. Louis, MO) to obtain $Srf^{f/f}$-GFAPCre+/- (Srf^{GFAP}CKO). $Srf^{f/f}$

154 mice served as control in all experiments. Both male and female mice were used in all the

155 experiments. All experiments were conducted in accordance with the animal care standards and

156 use and approved by the Institutional Animal Ethics Committee. Control and mutant mice were

157 housed together, and cage mates were randomly assigned to experimental groups. All

158 experiments were conducted blinded to the genotype of the mice used.

159

160 **Immunohistochemistry.** Mice were fixed by transcardial perfusion using 4% PFA. The brains

161 were cryoprotected in 30% sucrose, frozen and stored in -80° C until further use. For staining,

162 30 µm thick cryosections were incubated in blocking/permeabilization solution containing 0.3%

163 Triton-X and 3% goat serum in PBS (pH 7.4) for 1 hour followed by overnight incubation in

164 primary antibody. The brain sections were then washed in PBS and incubated in secondary

165 antibody for 1 hour. The sections were finally mounted in DAPI-containing mounting medium

166 (Vector Laboratories). For SRF immunostaining, heat-induced epitope retrieval was carried out

167 by incubating the sections in 10 mM sodium citrate, pH 8.9 for 30 min at 95 ºC. The following

168 primary antibodies were used: anti-GFAP (1:1000; Sigma-Aldrich Cat# G3893,

169 RRID:AB_477010), anti-GFAP (1:1000; Agilent Cat# Z0334, RRID:AB_10013382), anti-Nestin

6

170　(1:1000; Millipore Cat# MAB5326, RRID:AB_2251134), anti-Vimentin (1:50; DSHB Cat# 40E-

171　C, RRID:AB_528504), anti-S100β (1:1000; Sigma-Aldrich Cat# S2644, RRID:AB_477501),

172　anti-S100β (1:500; Synaptic systems Cat# 287006, RRID:AB_2713986), anti-Aldh1L1 (1:100;

173　UC Davis/NIH NeuroMab Facility Cat# 73-140, RRID:AB_10673447), anti-Iba1 (1:1000; Wako

174　Cat# 019-19741, RRID:AB_839504), anti-PhosphoHistoneH3 (1:500; Sigma-Aldrich Cat#

175　H0412, RRID:AB_477043), anti-Caspase 3 active (1:1500, Millipore Cat# 04-439,

176　RRID:AB_673061), anti-SRF (1:200; Santa Cruz Biotechnology Cat# sc-335,

177　RRID:AB_2255249) and anti-NeuN (1:500; Millipore Cat# MAB377, RRID:AB_2298772).

178　The following secondary antibodies were, AlexaFluor-488, -594, and -647 conjugated anti-

179　rabbit, anti-chicken, and anti-mouse at 1:1000 dilution (Life Technologies). Biotinylated anti-

180　mouse and anti-rabbit secondary antibodies (1:250; Vector Laboratories) were used along with

181　Vectastain ABC Elite, ImmPACT VIP substrate and ImmPACT DAB substrate kits (Vector

182　Labs). All the images were captured using conventional fluorescence microscopy (Eclipse 80i,

183　Nikon), except the images for SRF immunostaining and BBB measurement, which were

184　captured using a confocal microscope (LSM 880, Zeiss).

185

186　**RNA Isolation and quantitative realtime PCR.** Total RNA was isolated from forebrain of 3-5

187　wk old control and Srf^{GFAP}CKO mice using the PureLink™ RNA Mini Kit (Thermo Fisher

188　Scientific) as per the manufacturer's protocol. 2 µg of total RNA was used for first-strand cDNA

189　synthesis using the first strand synthesis kit (Invitrogen Inc.). Quantitative RT-PCR was done

190　with 100 ng of cDNA and KAPA SYBR FAST ABI prism kit (Cat. No. KK4604) using the

191　following program: 95° C or 3 min followed by 39 cycles of 95° C for 5 sec, 55° C for 30 sec and

192　72° C for 40 sec. The PCR reaction was carried out in QuantStudio 7 Flex Real-Time PCR

7

193   System (Invitrogen Biosciences). The primers used were: *Srf*, Fwd, 5'-

194   ACCAGTGTCTGCTAGTGTCAGC-3' and Rev, 5'-CATGGGGACTAGGGTACATCAT-3';

195   *Rps29*, Fwd 5'-CCAGCAGCTCTACTGGAGTCA-3' and Rev, 5'-

196   AGACTAGCATGATCGGTTCCA-3'. *Il1β*, Fwd, 5'-ATCAACAAGCAATTCCTCGATGA-3'

197   and Rev, 5'-CAGCATTCGCTTCAAGGACATA-3'; TNFa, Fwd, 5'-

198   CCCTCACACTCAGATCATCTTCT-3' and Rev, 5'-GCTACGACGTGGGCTACAG-3'; *Ccl2*,

199   Fwd, 5'-TTAAAAACCTGGATCGGAACCAA-3' and Rev, 5'-

200   GCATTAGCTTCAGATTTACGGGT-3'. Expression of *Srf* and other genes were normalized to

201   that of the housekeeping gene, *Rps29*. The primers for A1, A2 and pan-reactive astrocytes were

202   from a previously published study (Liddelow et al., 2017).

203

204   **BBB permeability assay**. Two assays were used as previously described (Andreone et al.,

205   2017) to measure the integrity of BBB using 6-mon old Srf^{GFAP}CKO mice. In the first assay,

206   mice were deeply anesthetized with isoflurane and injected with 20µl of 10 kDa dextran

207   fluorescein (10 mg/ml, Invitrogen; D1820) into the left ventricle of the heart, and allowed to

208   circulate for 5 min. Their brains were collected and post-fixed in 4% PFA overnight, frozen and

209   stored at -80° C. 30 µm thick cryosections were mounted using mounting media supplemented

210   with DAPI (Vector Labs, USA) and analyzed using a confocal microscope (LSM 880, Zeiss). In

211   the second assay, 10 µl of HRP Type II (5mg/ml) was administered transcardially and allowed to

212   circulate for 5 min. The brains were collected and immersed in 2% glutaraldehyde in 4% PFA in

213   cacodylate buffer (0.1M, pH 7.3) at RT for 1 hour. The brains were then shifted to 4° C

214   overnight and sectioned at 100 µm using a Leica vibratome, and processed using ImmPACT VIP

215   kit (Vector Labs). For quantification of dextran fluorescein injection, epifluorescence images

216 (63X) of brain sections were analyzed using ImageJ. Brain sections from the same rostro-caudal

217 position were analyzed. At least 12 different regions were taken and the ratio of the fluorescence

218 or color intensity (outside versus inside the vessel) was measured.

219

220 **Quantification of fluorescence intensity and cell numbers.** For measuring fluorescence

221 intensity, images were scaled for 10X magnification and normalized to the same exposure time.

222 Ten to twelve areas of field (ROI, 500x500 μm^2) in the same rostro-caudal axis were drawn per

223 image, and the intensities were measured using ImageJ (Fiji) after subtracting the background

224 fluorescence from both control and knockout sections. For the hippocampus, cell numbers or

225 fluorescence intensities were measured in the *stratum oriens* and *stratum radiatum*. For cell

226 counts, images were taken at 4X magnification. Four ROIs of 1200x1200 μm^2 (1 mm^2 for

227 hippocampal CA1 and CA3) in the same rostro-caudal axis were drawn per image, and the

228 number of cells per ROI were counted with cell counter plugin using ImageJ (Fiji). For SRF

229 fluorescence intensity, the SRF fluorescence signal within the DAPI area was quantified using

230 ImageJ.

231

232 **Quantification of microglial activation**. Brain sections from Srf^{GFAP}CKO mice and control

233 littermates at different ages (3 wk, 3 mon and 12 mon) were fluorescently immunostained for

234 Iba1. At least five different cortical regions in the same rostro-caudal axis were taken in all the

235 mice to measure fluorescence intensity. This was then compared between the control and the

236 knockout mice to get the fold difference in fluorescence. ImageJ was used to measure the

237 percentage increase in fluorescence per unit field and this was compared between control and

9

238 knockout groups at all the experimental time points as mentioned in statistical analysis. The area

239 of the field was 500x500 μm^2.

240

241 **TUNEL assay**. The TUNEL assay was carried out using Click-IT Plus TUNEL assay kit

242 (Molecular Probes, Thermo Fisher Scientific) according to the manufacturer's instructions.

243 Briefly, brains fixed in 4% PFA and 30 μm cryosections were permeabilized with proteinase K

244 solution for 15 min and then incubated with TdT reaction mixture for 60 min at 37° C and

245 subsequently with EdUTP for 30 min. The detection was achieved through click reaction

246 between the dUTP bound alkyne group and a picolyl azide fluorescent dye for 30 min. The slides

247 were washed with 3% BSA in PBS for 5 min and rinsed in 1X PBS. The slides were mounted

248 using mounting medium containing DAPI (Vector Labs) and observed using an epifluorescence

249 microscope (Eclipse 80i, Nikon) using appropriate filters and captured using Metamorph

250 software. Numbers of TUNEL$^+$ cells in the CA1, CA3 and DG regions of entire rostral to caudal

251 brain regions were counted using Image J software. The area of the field for counting the number

252 of TUNEL$^+$ cells was 250x250 μm^2.

253

254 **Statistical analyses**. Analyses were done using GraphPad Prism 6. The comparisons between

255 two groups was done using unpaired two-tailed Student's *t*-test. All the statistical details for each

256 experiment, including the n value, the statistical test used, P value, significance of comparisons is

257 mentioned in the figure legends.

258

259

260 **RESULTS**

10

261 ***Srf* deletion in astrocytes results in reactive astrogliosis**

262   To study the function of SRF in astrocytes, we generated *Srf*GFAPCKO conditional

263 knockout mice using a hGFAP-Cre transgenic mouse line, in which Cre expression was found to

264 occur predominantly in astroglial progenitor cells starting at embryonic day 16.5 (E16.5)

265 (Bajenaru et al., 2002). *Srf*GFAPCKO mice were obtained in normal Mendelian ratio, appeared

266 normal at birth and did not exhibit any gross morphological deficits such as neocortical

267 lamination and hippocampal architecture (data not shown). We first confirmed *Srf* deletion in

268 astrocytes. Co-immunostaining for SRF, S100β and NeuN revealed robust SRF expression in

269 both the astrocytes and neurons in 3-5 wk old control mice (Fig 1A). In the brain sections of

270 *Srf*GFAPCKO mutant mice, there was robust SRF expression in neurons while it was absent in the

271 astrocytes (Fig. 1 A, B). We observed that the antigen retrieval method caused a slightly

272 punctate SRF immunostaining, which was more pronounced in the mutant sections. However,

273 this pattern of staining overlapped with NeuN but not with S100β in the mutant sections (Fig.

274 1A). Quantitative and semi-quantitative real time PCR using total RNA isolated from whole

275 brain showed decreased *Srf* expression in mutants relative to controls (Fig. 1C, D).

276

277   We then asked whether *Srf* deletion had any effect on astrocyte development. In 3-5 wk

278 old control mice, astrocytes in hippocampus and fibrous astrocytes in the white matter expressed

279 GFAP, while there was no detectable GFAP expression in the neocortical astrocytes, which have

280 been shown to downregulate GFAP expression postnatally (Buffo et al., 2008; Robel et al., 2009)

281 (Fig. 2A ,B). However, the astrocytes in control mice expressed other astrocytic markers, such

282 as S100β (Fig. 2C). In striking contrast, astrocytes in the *Srf*GFAPCKO mice exhibited

283 pronounced GFAP expression (Fig. 2A, B) along with hypertrophic morphology as seen from

11

284   immunostaining for GFAP, Aldh1L1 and S100β (Fig. 2A-C), both hallmarks of reactive

285   astrogliosis. Cell counts of S100β-positive cells revealed no change in the number of astrocytes

286   between control and mutant mice in all regions analyzed (Fig. 2D). We had shown earlier that

287   neuron-specific deletion of SRF does not affect astrocyte differentiation or cause reactive gliosis

288   (Lu and Ramanan, 2012). Therefore, although unlikely, any transient Cre expression in neurons

289   by the hGFAP-Cre transgene is unlikely to cause the gliosis phenotype seen in SrfGFAPCKO

290   mice.

291

292   Astrocytes exhibit regional heterogeneity and previous gene deletion studies have shown

293   region-specific generation of reactive astrocytes (Garcia et al., 2010; Kang et al., 2014b). We

294   therefore asked whether reactive astrocytes in Srf^{GFAP}CKO mice were also regionally restricted.

295   For this, we analyzed serial sections from the entire brain of control and Srf knockout mice. We

296   found that astrocytes in most regions of the brain in control mice did not express or weakly

297   expressed GFAP (Fig. 2A, B). In striking contrast, brain sections from Srf^{GFAP}CKO mice

298   exhibited intense GFAP expression in all brain regions analyzed including in striatum and corpus

299   callosum (data not shown). To further confirm that the astrocytes in Srf^{GFAP}CKO mice are

300   indeed reactive, we immunostained for known astrogliosis markers, vimentin and nestin (Ridet et

301   al., 1997). In contrast to control littermates, the Srf^{GFAP}CKO mice exhibited robust nestin-

302   positive (Fig. 3A, B) and vimentin-positive (Fig. 3C, D) astrocytes, thus confirming their

303   reactive state. These observations suggest that astrocyte-specific deletion of Srf results in

304   reactive astrocytes.

305

306   **Astrogliosis seen in *Srf* knockout mice is not induced by cell death.**

12

307   Astrogliosis is generally induced by several extrinsic factors, such as cell death or a leaky

308   blood brain barrier (BBB) (Pekny and Nilsson, 2005). We first analyzed cell death by

309   immunostaining for cleaved caspase-3 as well as by TUNEL staining. We did not observe any

310   discernible cell death at 2 weeks of age in Srf^{GFAP}CKO mice, just prior to the onset of

311   astrogliosis (Fig. 4A-C). This strongly suggested that reactive astrogliosis seen in the Srf

312   knockout mice is not triggered by cell death. During conditions of severe neural injury or

313   trauma, reactive astrocyte undergo proliferation (Sofroniew, 2014). To determine whether SRF-

314   deficient reactive astrocytes are proliferating, brain sections from Srf^{GFAP}CKO mice and control

315   littermates were immunostained with the proliferation marker, phosphoHistone-H3 (phH3).

316   There were no phosphoHistone-H3-positive cells observed in either Srf mutant mice or their

317   control littermates, excluding the presence of proliferating astrocytes (Fig. 4D).

318

Blood brain barrier is unaffected in *Srf* knockout mice

320   Given the widespread and persistent reactive astrogliosis seen in Srf mutant mice, it is

321   possible that a leaky blood brain barrier (BBB) could be the likely cause for the astrogliosis

322   observed in Srf knockout mice (Pekny and Nilsson, 2005). To study BBB integrity, we injected

323   two different tracers, 10 kDa dextran fluorescein (Fig. 5A, B, C) and 44 kDa HRP type II (Fig.

324   5A, D, E), into 3-5 week old Srf^{GFAP}CKO mutant and control mice (Andreone et al., 2017). We

325   did not observe any discernible presence of these tracers in the brain parenchyma of Srf knockout

326   mice relative to their littermate controls (Fig. 5), supporting an intact BBB.

327

Persistent reactive astrogliosis seen in *Srf*^{GFAP}CKO mice throughout adulthood

13

329   Astrogliosis could be a transient phenomenon, lasting a few days to several weeks, or a

330   long-lasting event resulting in a glial scar, depending on the severity of trauma or injury

331   (Sofroniew, 2014). We therefore asked whether the astrogliosis seen in Srf^{GFAP}CKO mice is a

332   transient process. To address this, we immunostained brain sections from 3-month and 12-

333   month old control and knockout mice. The astrocytes in hippocampus of control mice showed

334   normal GFAP expression while there was very faint or no GFAP expression in the astrocytes in

335   the other brain regions both at either 3 month (Fig. 6A, B) or 12 months of age (Fig. 6C, D). In

336   contrast, the astrocytes in the knockout mice, at both 3 and 12 months of age, expressed intense

337   GFAP expression and exhibited hypertrophy in all brain regions (Fig. 6A-D) similar to that seen

338   at 3 weeks (Fig. 2, 3). To further confirm astrogliosis, we immunostained for vimentin and

339   found robust expression only in the astrocytes in the knockout mice but not in their control

340   littermates (Fig. 6E-G). Immunostaining for phosphoHistone-H3 did not reveal any positive

341   cells, suggesting that these astrocytes are also not proliferative in older mice (Fig. 6H). Together

342   these observations demonstrate that SRF ablation in astrocytes results in widespread astrogliosis

343   that persists through adulthood.

344

Microglial activation in Srf^{GFAP}CKO mice

346   Reactive astrocytes are often observed along with microgliosis (Frank and Wolburg,

347   1996; Zhang et al., 2010). We therefore asked whether microglia exhibited a reactive state in the

348   Srf knockout mice. Immunostaining for the microglial marker, Iba1 showed increased

349   expression in brain sections from 3-wk old mutant mice, relative to control littermates, indicative

350   of reactive microglia (Fig. 7A, B). We then quantified the number of Iba1$^+$ cells and found no

351   significant difference in microglial numbers between control and knockout mice (Fig. 7C).

14

352   However, we noticed that the microglia tend to form clusters in the cortex and hippocampus

353   (Fig. 7A). We next asked whether microgliosis also persisted throughout adulthood similar to

354   that seen for astrogliosis. As seen at 3 weeks, brain sections from 3-month and 12-month old

355   Srf^{GFAP}CKO mice also showed strong upregulation of IbaI expression relative to that from

356   control mice (Fig. 7D, E) suggesting that microglia also exhibited a reactive state along with

357   reactive astrocytes. Similar to that seen at 3 wks of age, we did not find any difference in the

358   number of Iba1$^+$ cells in both 3-mon and 12-mon old Srf^{GFAP}CKO mice compared to control

359   littermates (Fig. 7F, G). Immunostaining for proliferation marker, phosphoHistone-H3 did not

360   reveal any positive cells (Fig. 4D, 6H) indicating that these reactive microglia are also not

361   proliferative.

362

363   **Persistent gliosis in Srf^{GFAP}CKO mice does not affect neuronal viability**

364   Recent studies have shown that reactive astrocytes can be broadly classified as either

365   neurotoxic (A1) or neuroprotective (A2) depending on the external stimuli (Zamanian et al.,

366   2012; Liddelow et al., 2017). In order to determine the phenotypic state of SRF-deficient

367   reactive astrocytes, we performed quantitative RT-PCR for some of the A1 (*Psmb8, H2T23,*

368   *H2D1, Srgn*), A2 (*Cd109, Ptgs2, Clcf1, Cd14*) and pan-reactive marker genes (*Serpina3n, Gfap*).

369   Although we found that the Srf^{GFAP}CKO mice expressed both A1 and A2 genes, there were more

370   A2 reactive astrocyte marker genes that were upregulated compared to A1 genes suggesting that

371   the SRF-deficient reactive astrocytes are likely to be A2-like (Fig. 8A). Since we also observed

372   microgliosis, we next assessed the expression of neuroinflammatory genes. Quantitative PCR

373   showed a significant increase in the expression of *Il1β* and *Ccl2/Mcp-1* but not *TNFα* in the

374   brains of Srf^{GFAP}CKO mice (Fig. 8A) suggesting that these genes could be one of the underlying

375   causes of microgliosis observed in the *Srf* mutant mice.

376

377   We next asked whether prolonged gliosis in the SRF-GFAP mutant mice affected

378   neuronal numbers. For this, 5-wk and 12-mon old brain sections from Srf^{GFAP}CKO mice and

379   control littermates were immunostained for the neuronal marker, NeuN. We did not observe any

380   significant difference in NeuN-positive cells in the brains of mutant and control mice at both

381   these ages (Fig. 8B-D). This suggested that persistent gliosis observed in the brains of

382   Srf^{GFAP}CKO mice did not affect neuronal survival and that SRF-deficient reactive astrocytes are

383   likely not neurotoxic.

384

385   **DISCUSSION**

386   Reactive astrogliosis is an important cellular response to neuronal injury, infection and

387   neurodegeneration in the CNS, and this is critical to reduce inflammation, restrict tissue damage

388   and cell death, and promote tissue repair (Sofroniew, 2005; Pekny and Pekna, 2014). Currently,

389   very little is known about the cell-intrinsic mechanisms that regulate the conversion of an

390   astrocyte from a non-reactive to a reactive state. In this study, we show that deletion of the

391   transcription factor, SRF in astrocytes results in widespread reactive gliosis in the brain starting 3

392   weeks of age. The reactive astrocytes persisted along with microgliosis throughout adulthood

393   and both astrocytes and microglia did not exhibit proliferation. Our results suggest that SRF is

394   required in a cell-autonomous manner to regulate reactive astrogliosis in the mammalian brain.

395

16

396   SRF is a ubiquitously expressed transcription factor that has been shown to play critical

397   roles in several aspects of nervous system development and function (Knoll and Nordheim,

398   2009).   Deletion of SRF in developing and adult neurons resulted in deficits in neuronal

399   migration, axon growth, hippocampal circuit formation, activity-dependent gene expression and

400   learning and memory (Alberti et al., 2005; Ramanan et al., 2005; Etkin et al., 2006; Knoll et al.,

401   2006; Wickramasinghe et al., 2008; Stritt and Knoll, 2010; Johnson et al., 2011; Lu and

402   Ramanan, 2011; Li et al., 2014).   Deletion of SRF within neural stem cells specifically affected

403   differentiation to both astrocytes and oligodendrocytes (Lu and Ramanan, 2012).   Interestingly,

404   neuronal SRF deletion did not affect astrocyte differentiation or cause any reactive astrogliosis

405   (Lu and Ramanan, 2012) but revealed a paracrine effect of neuronal SRF on oligodendrocyte

406   maturation and myelination (Stritt et al., 2009; Anastasiadou et al., 2015). Currently, the role of

407   SRF in astrocyte development remains poorly understood.   This study identifies a critical role for

408   SRF in maintenance of astrocytes in a non-reactive state.   However, it is possible that since SRF

409   deletion in the Srf^{GFAP}CKO mice likely starts around E16.5 during embryonic development, the

410   reactive astrogliosis could be due to developmental deficits.

411

412   Previous studies have shown that genetic ablation of the extracellular matrix protein, β1-

413   integrin (Itgβ1) in astrocytes results in astrogliosis starting 4 weeks of age and Itgβ1 mutant mice

414   exhibit spontaneous seizures (Robel et al., 2009; Robel et al., 2015).   Attenuation of sonic

415   hedgehog (Shh) signaling in postnatal astrocytes resulted in reactive astrocytes that were

416   restricted to the forebrain alone suggesting a role for Shh in maintaining the non-reactive state of

417   specific astrocytic populations (Garcia et al., 2010).   Similarly, attenuation of fibroblast growth

418   factor (FGF) signaling by deletion of FGF receptors, FGFR1-3, resulted in astrogliosis that was

17

419   restricted to the neocortex and hippocampus although deletion occurred in other regions as well

420   (Kang et al., 2014b). Interestingly, expression of a dominant negative FGFR3 (dnFGFR3) and a

421   constitutively active FGFR3 (caFGFR3) in astrocytes produced different results.   While

422   caFGFR3 suppressed astrogliosis in one study, it resulted in enlarged astrocytes with increased

423   branching in another (Kang et al., 2014a; Kang et al., 2014b). Furthermore, expression of a

424   dnFGFR3 suppressed GFAP expression and hypertrophic morphology (Kang et al., 2014a).

425   Nevertheless, these observations suggest an important role for FGF signaling in regulating the

426   reactive state of forebrain astrocytes. Since reactive astrocytes can either be beneficial or

427   detrimental to normal functioning of the nervous system, this process is expected to be tightly

428   regulated to maintain astrocytes in a non-reactive state.

429

430   Reactive astrocytes are induced or regulated by several extracellular signals including

431   cytokines, growth factors, endothelin, purines and lipopolysaccharide (LPS) and these factors are

432   released by neural and non-neural cells in the CNS following neuronal injury or in response to

433   infection (Kang and Hebert, 2011; Sofroniew, 2014). In the Srf^{GFAP}CKO mice, we did not

434   observe any cell death when analyzed at 2 weeks of age (Fig. 4A-C) and therefore, cell death is

435   unlikely to be the cause of gliosis, which was first observed starting at 3 weeks of age.

436   Furthermore, there were no discernible deficits observed in BBB permeability indicating that

437   reactive astrocytes in the Srf mutant mice are not induced by a leaky BBB (Fig. 5).

438

439   Depending on the severity of neural injury, astrogliosis can manifest as mild, moderate or

440   severe without or with scar formation (Pekny and Pekna, 2014; Sofroniew, 2014). During mild

441   and moderate astrogliosis, astrocytes exhibit hypertrophy, do not proliferate and return to non-

18

442   reactive state once the underlying cause is resolved (Pekny and Pekna, 2014; Sofroniew, 2014).

443   In severe astrogliosis, astrocytes exhibit proliferation and can also result in scar formation with

444   significant tissue rearrangement. In the Srf^{GFAP}CKO, the astrocytes exhibited hypertrophy but

445   were not proliferative when assessed in young and old mice (Fig. 4D, 6H). The absence of

446   proliferative hypertrophic reactive astrocytes has been reported in mutant mice carrying

447   astrocyte-specific deletion of genes such as Sonic hedgehog (Garcia et al., 2010), β1-integrin

448   (Robel et al., 2009) and FGFR (Kang et al., 2014). Furthermore, the astrogliosis in these mutant

449   mice was found to be restricted to specific regions in the brain likely reflective of their

450   requirement in specific astrocyte populations. In contrast, in the Srf knockout mice, we observed

451   widespread astrogliosis in all regions of the brain including white matter astrocytes (Fig. 6A-G).

452   This suggests that conditional Srf ablation in astrocytes results in conversion to reactive

453   astrocytes irrespective of their regional heterogeniety (Zhang and Barres, 2010).

454

455   The Srf mutant mice also exhibited reactive microglia starting around 3 weeks of age and

456   persisted throughout adulthood (Fig. 7). Reactive astrogliosis has been shown to accompany

457   reactive microglia and vice versa (Frank and Wolburg, 1996; Zhang et al., 2010). Reactive

458   astrocytes are known to secrete cytokines, which can cause the activation of microglia (Davalos

459   et al., 2005). Since there appears to be no injury or infection in Srf^{GFAP}CKO mice, the microglial

460   activation is very likely to be caused by the reactive astrocytes.

461

462   Studies have shown that depending on the kind of neuronal injury or insult, reactive

463   astrocytes can be broadly classified as neurotoxic (A1) or neuroprotective (A2) (Li et al., 2008;

464   Zamanian et al., 2012; Liddelow et al., 2017). Since the SRF-deficient astrocytes exhibited

19

465 hypertrophy and enhanced GFAP and vimentin expression (Fig. 2, 3), we look at the expression

466 of A1 and A2 astrocyte markers. We found expression of more A2 marker genes as compared to

467 A1 genes in the brains Srf^{GFAP}CKO mice suggesting that SRF-deficient reactive astrocytes are

468 likely to be A2-type (Fig 8A). A1 reactive astrocytes have been shown to secrete neurotoxic

469 factor(s) that resulted in neuronal cell death in cultured neurons (Liddelow et al., 2017). The

470 presence of reactive microglia suggested that the reactive astrocytes could induce microgliosis.

471 We found an increased expression of *Il1β* and *Ccl2/Mcp-1* but not *TNFα* in brains of

472 Srf^{GFAP}CKO mice (Fig. 8A). IL-1β and the chemokine, CCL2/MCP-1 have been shown to

473 induce reactive microglia and could be the underlying cause for prolonged microgliosis (Selenica

474 et al., 2013; Liu and Quan, 2018). Since the Srf^{GFAP}CKO mice exhibited prolonged gliosis, we

475 analyzed for neuronal numbers and found no significant change in the number of neurons

476 indicating that the reactive astrocytes in the brains of these *Srf* mutant mice do not affect

477 neuronal survival (Fig. 8A-D).

478

479   In summary, we have identified SRF as a critical regulator of reactive astrocytes in the

480 mouse brain. Ablation of *Srf* in astrocyte-specific manner results in persistent and widespread

481 astrogliosis. Since astrocytes in all brain regions become reactive, it strongly suggests that SRF

482 is important for maintaining both white matter and grey matter astrocytes in a non-reactive state.

483 The SRF-deficient reactive astrocytes appear to be A2-like and the brains of Srf^{GFAP}CKO mice

484 exhibited normal neuronal numbers in spite of persistent gliosis. It is reasonable to speculate that

485 SRF expression needs to be downregulated for an astrocyte to become reactive. It is also

486 possible that SRF-dependent transcription is likely affected in reactive astrocytes observed in

487 neurodegenerative disorders. Future studies aimed at identification of SRF target genes may

488   provide novel insights into the mechanisms regulating reactive astrogliosis and may provide

489   potential targets for astrocyte-targeted therapeutics.

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592

593   **FIGURE LEGENDS**

594

595   **Figure 1.** Astrocyte-specific deletion of *Srf* in *Srf*^{GFAP}CKO mice. (**A**) Representative images of

596   immunostaining for SRF (red), S100β (green) and NeuN (blue) shows SRF expression in

597   astrocytes (arrows) and neurons (arrowheads) in the cortex and striatum of control mice. SRF

598   expression was seen only in the neurons but not in the astrocytes in *Srf*^{GFAP}CKO mice mutant

599   mice. **A'** shows representative higher magnification images of astrocytes and neurons from (A)

600   showing SRF expression in control astrocytes and neurons, and absent in mutant astrocytes. (B)

601   Quantification of SRF immunofluorescence in S100β+ astrocytes from (A). At least 15-20 cells

602   per mouse were analyzed (n=3 mice). Astrocytes: cortex, control (16.49±2.52), *Srf*^{GFAP}CKO

603   (4.80±0.29); Striatum, control (19.72±3.179), *Srf*^{GFAP}CKO (7.40±1.47); Neurons: cortex, control

604   (24.33±5.05), *Srf*^{GFAP}CKO, control (28.47±5.60); Striatum, control (30.79±1.40), *Srf*^{GFAP}CKO

605   (23.41±3.09). (C) Quantitative real time PCR from whole brain total RNA shows a significant

606   decrease in Srf mRNA expression in the mutant mice relative to control mice; Control (1.0±0),

607   *Srf*^{GFAP}CKO (0.30±0.14) (n=3 mice). (D) Semi-quantitative PCR from whole brain total RNA

608   shows a significant decrease in Srf mRNA expression in the mutant mice. Rps29 expression

609   served as the loading control (n=3 mice). Control (1.04±0.01), *Srf*^{GFAP}CKO (0.24±0.02) (n=3

610   mice). Scale bars, 50 μm (A), 20 μm (A'). ** P < 0.005, *** P < 0.0005, **** P < 0.0001, ns,

611   not significant. Two tailed t-test. Data are mean ± SEM.

612

613   **Figure 2.** Conditional deletion of SRF in astrocytes results in reactive gliosis. (**A**) Representative

614   images of immunostaining for GFAP and ALDH1L1 in 3-wk old *Srf*^{GFAP}CKO and control

615   littermates shows higher expression of these markers in neocortex (Ctx) and hippocampus (Hc)

26

616 of mutant mice as compared to control littermates. (**B, C**) GFAP and S100β immunostaining

617 also showed that the astrocytes in mutant mice were hypertrophic compared to control mice,

618 (n=3 mice). (**D**) Quantification of S100β$^+$ astrocytes from (C). Cortex, control (76.58±1.19),

619 *Srf*^{GFAP}CKO (50.25±5.67); Striatum, control (70.71±3.61), *Srf*^{GFAP}CKO (66.04±3.72);

620 Hippocampus, control (11.90±0.17), *Srf*^{GFAP}CKO (10.88±1.14) (n=3 mice). Scale bars, 100 μm

621 (**A**), 50 μm (**B, C**); ns, not significant. Two tailed *t*-test. Data are mean ± SEM.

622

623 **Figure 3.** SRF ablation in astrocytes leads to widespread astrogliosis. (**A**) Representative images

624 of immunostaining for the astrogliosis marker, nestin in 3-wk old *Srf*^{GFAP}CKO and control

625 littermates shows reactive astrocytes in cortex and hippocampus of *Srf* mutant mice but not in

626 control littermates. (**B**) Quantification of nestin fluorescence intensity shown in (A). Cortex:

627 control (0.0±0.14), *Srf*^{GFAP}CKO (771.9±16.14), hippocampus: control (0.0±8.15), *Srf*^{GFAP}CKO

628 (556.1±38.56) (n=3 mice). (**C**) Representative images showing co-immunostaining with GFAP

629 and the gliosis marker, vimentin. There is little or no GFAP and vimentin expression in 3-wk

630 control mice. In contrast, the astrocytes in mutant mice exhibit strong expression and

631 colocalization of GFAP and vimentin. (**D**) Quantification of fluorescence intensity in (C). For

632 GFAP, cortex, control (102.1±5.28), *Srf*^{GFAP}CKO (580.1±62.10); hippocampus, control

633 (228.0±34.20), *Srf*^{GFAP}CKO (638.1±73.97); striatum, control (85.78±1.83), *Srf*^{GFAP}CKO

634 (256.9±7.73); For vimentin, cortex, control (2.93±0.99), *Srf*^{GFAP}CKO (674.10±27.70);

635 hippocampus, control (12.02±12.02), *Srf*^{GFAP}CKO (964.10±11.79); striatum, control (2.33±0.33),

636 *Srf*^{GFAP}CKO (633.50±41.37). Shown here are neocortex, hippocampus (Hpc) and striatum (Str)

637 (n=3 mice). Scale bar, 500 μm (**A**), 50 μm (**C**). ** P < 0.005, *** P < 0.0005, **** P < 0.0001,

638 Two tailed *t*-test. Data are mean ± SEM.

27

639

640   **Figure 4.** Absence of cell death in *Srf* mutant mice. (**A**) Representative images of TUNEL

641   staining of 2-wk old *Srf*^{GFAP}CKO mice and control littermates. Amplified view of boxed region

642   is shown on right. Arrows show TUNEL$^+$ cells while the arrowhead shows non-specific

643   staining. (**B**) Representative images of immunostaining for cleaved caspase-3 in the neocortex

644   and hippocampus of 2-wk old *Srf*^{GFAP}CKO mice and control littermates. Amplified view of

645   boxed region is shown on right. Arrows show TUNEL$^+$ cells while the arrowhead shows non-

646   specific staining. (**C**) Quantification of TUNEL$^+$ and cleaved Caspase-3$^+$ cells in neocortex and

647   hippocampus shows the no significant difference in the number of dead cells between

648   *Srf*^{GFAP}CKO mice and control littermates. Caspase-3: cortex, control (2.20±1.00), *Srf*^{GFAP}CKO

649   (1.45±0.77); hippocampus, control, (1.16±0.65), *Srf*^{GFAP}CKO (0.91±0.79). TUNEL: cortex,

650   control (0.37±0.21), *Srf*^{GFAP}CKO (0.08±0.052); hippocampus, control (0.81±0.21), *Srf*^{GFAP}CKO

651   (1.18± 0.25) (n=3 mice). (**D**) Representative images of immunostaining for the proliferation

652   marker, phosphohistone H3 (phH3) in 3-wk old *Srf*^{GFAP}CKO and their respective control mice

653   showed no proliferating cells in the mutant mice. Immunostaining of P0.5 mouse brain section

654   showed many phH3-positive cells and served as a control. n.s, not significant. Two-tailed t-test.

655   Data are mean ± SEM. Scale bar, 20 µm (**A', B'**) and 200 µm; (n=3 mice).

656

657   **Figure 5.** Blood brain barrier is not compromised in *Srf*^{GFAP}CKO mice. (**A**) Schematic diagram

658   showing experimental timeline of dextran fluorescein and HRP injection and tissue processing.

659   (**B**) 10 kDa TMR-Dextran (Dextran fluorescein, DF) tracer injection reveals normal architecture

660   of cerebral vasculature in 3- to 5-wk old *Srf*^{GFAP}CKO and their control littermates. (**C**)

661   Quantification of ratio of fluorescence intensity outside vs inside the blood vessel reveals no

662   significant difference between Srf^{GFAP}CKO mice and their control littermates, indicative of intact

663   blood brain barrier. A stab-wounded brain served as a control to show BBB leakage. Control

664   (0.21±0.0), Srf^{GFAP}CKO (0.18±0.05), Stab-wound (1.97±0.26) (n=3 mice). (**D**) Transcardial

665   injection of 44 kDa HRP type II in Srf^{GFAP}CKO mice and their respective controls, shows that

666   HRP was restricted to the blood vessel lumen. (**E**) Quantification of ratio of color intensity

667   outside vs inside the blood vessel shows no significant difference between control and mutant

668   mice (n=3 mice). Control (0.16±0.05), Srf^{GFAP}CKO (0.20±0.02), Stab-wound (1.45±0.08) (n=3

669   mice). Scale bar, 400 μm (**B**), 200 μm (**D**). ns, not significant. **** P < 0.0001, one-way

670   ANOVA, data are mean ± SD.

671

672   **Figure 6**. Persistent astrogliosis in Srf^{GFAP}CKO mice. (**A**) Immunostaining for GFAP in 3-

673   month old Srf^{GFAP}CKO mice and control littermates shows widespread astrogliosis in all the

674   brain regions. (**B**) Quantification of color intensity in (A). Cortex, control (107.2±0.61),

675   Srf^{GFAP}CKO (176.4±7.35); hippocampus, control (270.2±9.11), Srf^{GFAP}CKO (385.4±16.14);

676   striatum, control (151.4±1.280), Srf^{GFAP}CKO (260.6±18.58) (n=3 mice). (**C**) Representative

677   images of immunostaining for GFAP in 12-month old Srf^{GFAP}CKO mice and control littermates

678   shows persistent astrogliosis in all the brain regions analyzed. Shown here are cortex,

679   hippocampus (Hpc) and striatum. (**D**) Quantification of relative color intensity in (C).

680   Quantification of color intensity in (C); cortex, control (188.2±2.85), Srf^{GFAP}CKO

681   (536.0±26.03); hippocampus, control (356.3±6.89), Srf^{GFAP}CKO (591.0±29.43); striatum: control

682   (191.1±4.83), Srf^{GFAP}CKO (495.7±80.51) (n=3 mice). (**E**) Representative images of

683   immunostaining for reactive astrogliosis marker, vimentin, in 3-mon and 12-mon old

684   Srf^{GFAP}CKO mice and control littermates shows gliosis astrocytes only in the brains of *Srf*

685   mutant mice. (**F, G**) Relative fluorescent intensity of vimentin immunostaining in 3-month (F)

686   and 12-month (G) old mice compared to control littermates. (F) Cortex, control, (11.52±11.52),

687   Srf^{GFAP}CKO (317.1±39.27); hippocampus: control (4.47±3.11), Srf^{GFAP}CKO (378.7±8.17),

688   striatum: control (5.43±5.43), Srf^{GFAP}CKO (110.8±4.94). (G) Cortex, control (8.22±4.11),

689   Srf^{GFAP}CKO (475.7±67.75); hippocampus, control (10.26±10.26), Srf^{GFAP}CKO (531.6±31.96);

690   striatum: control (17.30±11.98), Srf^{GFAP}CKO (157.4±13.96) (n=3-4 mice). (**H**) Immunostaining

691   for the proliferation marker, phosphohistone H3 (phH3) in 12-month old Srf^{GFAP}CKO and

692   control littermates showed no proliferating cells even at this age. Scale bar, 200 μm. * P < 0.05,

693   ** P < 0.005, *** P < 0.0005, **** P < 0.0001, Two tailed *t*-test. Data are mean ± SEM.

694

695   **Figure 7.** Microglial activation is seen along with astrogliosis in Srf^{GFAP}CKO mice. (**A**)

696   Immunostaining for the microglia marker, Iba1 showed increased Iba1 expression in the

697   neocortex and hippocampus of mutant mice as compared to the control littermates, suggesting

698   activated microglia in mutant brain. (**B**) Relative fluorescence intensity of Iba1 in the neocortex

699   of *Srf* mutant mice compared to control littermates in (A) shows a significant increase in Iba1

700   expression in the *Srf* mutants indicative of microgliosis. Control (1.0±0.0), Srf^{GFAP}CKO

701   (1.97±0.19) (n=3 mice). (**C**) Quantification of Iba1$^+$ cells in 3-wk old control and mutant mice.

702   Cortex, control (29.08±0.43), Srf^{GFAP}CKO (34.4±2.68); hippocampus (Hpc), control

703   (28.64±0.82), Srf^{GFAP}CKO (33.80±1.40); striatum, control (29.46±1.20), Srf^{GFAP}CKO

704   (32.0±0.42) (n=3 mice). (**D**) Representative images of sections immunostained for microglial

705   marker Iba1 in aged mice (3-month and 12-month old) in Srf^{GFAP}CKO and control littermates

706   show increased expression of Iba1 in the *Srf* mutants. Shown here is neocortex. (**E**) Relative

707   fluorescence intensity of Iba1 in the neocortex of *Srf* mutant mice compared to control

708   littermates shows persistent microgliosis in the *Srf* mutants throughout adulthood. 3 mon, control

709   (1.0±0.0), *Srf*^{GFAP}CKO (1.8±0.22); 12 mon, Control (1.0±0.0), *Srf*^{GFAP}CKO (1.71±0.17) (n=3

710   mice). (**F, G**) Quantification of Iba1$^+$ cells at 3 mon (F), cortex, control (30.00±0.62),

711   *Srf*^{GFAP}CKO (54.50±6.87); hippocampus (Hpc), control (24.00±4.25), *Srf*^{GFAP}CKO (58.75±9.00);

712   striatum, control (29.25±1.25), *Srf*^{GFAP}CKO (32.75±3.00); and 12 mon (G) cortex, control

713   (28.25±0.57), *Srf*^{GFAP}CKO (38.75±2.49); hippocampus, control (27.58±1.55), *Srf*^{GFAP}CKO

714   (36.75±6.00); striatum, control (28.17±0.88), *Srf*^{GFAP}CKO (28.58±1.17). (n=3 mice), Scale bars,

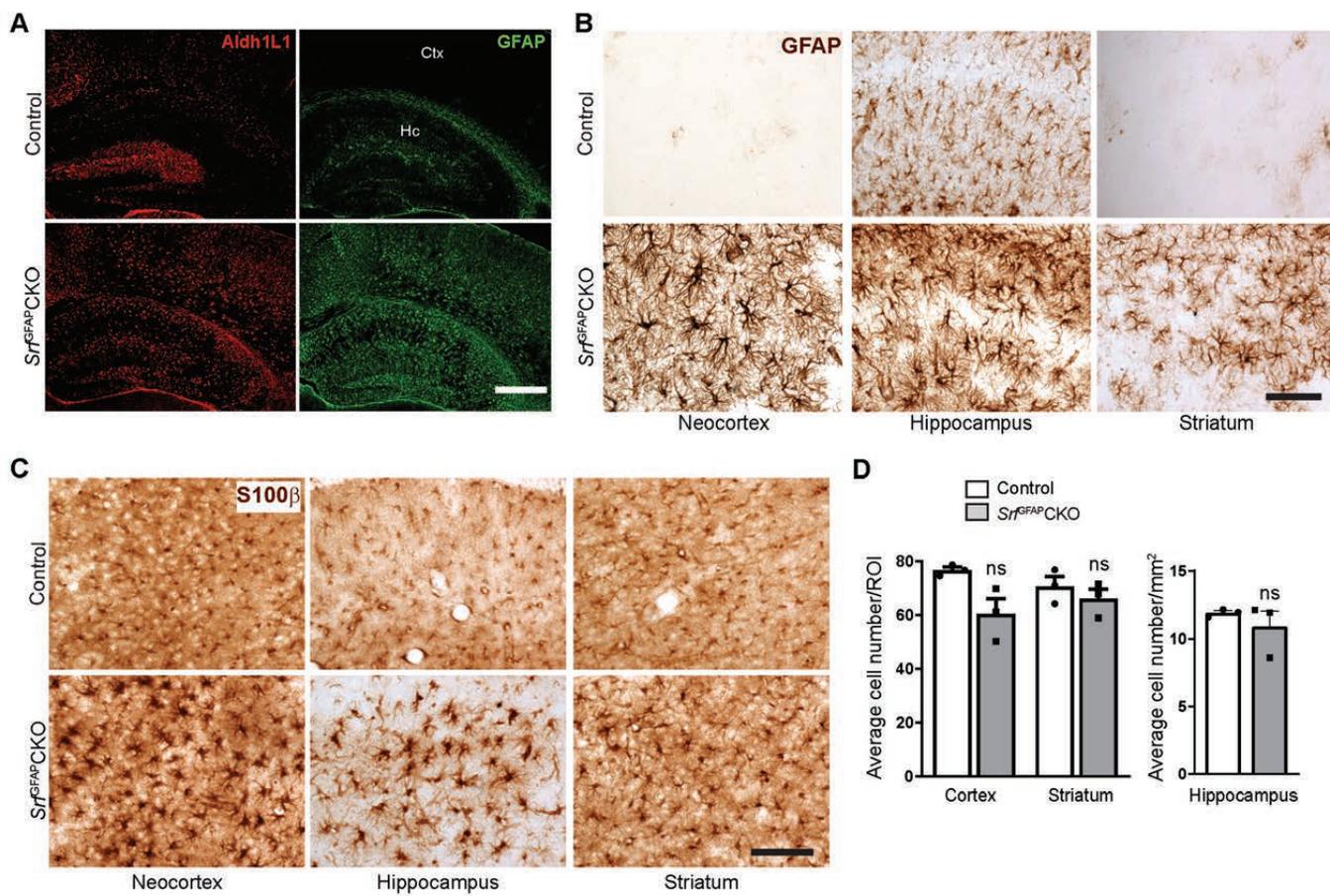715   200 μm; ** $P < 0.005$. ns, not significant. Two tailed t-test. Data are mean ± SEM.

716

717   **Figure 8.** Prolonged gliosis in *Srf*^{GFAP}CKO mice does not affect neuronal survival. (**A**)

718   Quantitative realtime PCR for A1, A2, pan reactive astrocyte markers and neuroinflammatory

719   genes shows expression of a greater number of A2 reactive genes in the brains of *Srf* mutant

720   mice compared to A1 genes. The mutant astrocytes also exhibited a higher expression of

721   neuroinflammatory genes, *Il1β* and *Ccl2/Mcp-1* but not *TNFα*. Control (1.0±0.0), *Srf*^{GFAP}CKO,

722   *Cd109* (3.49±0.62)*, Clcf1* (2.51±0.52)*, Cd14* (2.82±0.51)*, Ptgs2* (0.90±0.25), *Psmb8*

723   (7.43±0.83), *H2T23* (2.20±0.78), *H2D1* (2.4±0.65), *Srgn* (1.78±0.39), *Serpina3n* (10.88±1.86),

724   *Gfap* (9.62±0.84), *Il1β* (1.806±0.16), *Ccl2/Mcp-1*(13.52±1.967), *TNFα* (3.35±0.97) (n=3 mice).

725   (**B**) Representative images of immunostaining for the neuronal marker, NeuN shows normal

726   structural integrity in 3-5 wk old *Srf*^{GFAP}CKO mice compared to control littermates. (**C, D**)

727   Quantification of NeuN$^+$ cells in 3-5 wk old (C) and 12-mon old (D) control and mutant mice

728   shows no significant change in neuronal numbers in the mutant mice. (C) cortex, control

729   (209.5±12.10), *Srf*^{GFAP}CKO (195.2±11.18); CA1, control (78.25±16.48), *Srf*^{GFAP}CKO

730   (148.4±34.10); CA3, control (124.4±24.47), *Srf*^{GFAP}CKO (133.5±37.20); striatum, control

31

731   (220.8±5.90), *Srf*^{GFAP}CKO (205.8±19.59) (n=3 mice). (D) cortex, control (208.4±9.87),

732   *Srf*^{GFAP}CKO (190.0±9.71); CA1, control (119.0±22.36), *Srf*^{GFAP}CKO (148.2±12.23); CA3,

733   control (101.0±22.05), *Srf*^{GFAP}CKO (127.8±12.19); striatum, control (211.1±5.14), *Srf*^{GFAP}CKO

734   (182.1±51.89) (n=3 mice), Scale bar, 1 mm; * P < 0.05, ** P < 0.005, *** P < 0.0005, ns, not

735   significant. Two tailed t-test. Data are mean ± SEM.

736

Figure 2

A Aldh1L1 | GFAP
Control | Ctx | Hc
Srf^GFAP CKO

B GFAP
Control
Srf^GFAP CKO
Neocortex | Hippocampus | Striatum

C S100β
Control
Srf^GFAP CKO
Neocortex | Hippocampus | Striatum

D Control | Srf^GFAP CKO

A

Hippocampus | Cortex

Nestin DAPI

Control

Srf^{GFAP}CKO

B

900

600

300

0

Cortex | Hippocampus

anti-Nestin relative fluorescent intensity (arbitrary units)

□ Control
■ Srf^{GFAP}CKO

**

**

Figure 3

C

DAPI | GFAP | Vim | Merge

Control
Cortex
Hippocampus
Striatum

Srf^{GFAP}CKO
Cortex
Hippocampus
Striatum

D

□ Control
■ Srf^{GFAP}CKO

900

600

300

0

Cortex | Hpc | Str

anti-GFAP relative fluorescence intensity (arbitrary units)

****

**

***

1000

800

600

400

200

0

Cortex | Hpc | Str

anti-Vimentin relative fluorescence intensity (arbitrary units)

**

***

**

Figure 4

Figure 5

A

Transcardial injection → 5 min → Euthanize → 2 days → Section

Dextran Fluorescein -10 kDa or HRP Type II

PFA fixing and sucrose protection

B

DF | Control | Srf^{GFAP}CKO | Stab-wound

C

Fluorescence intensity (outside/inside)

Control | Srf^{GFAP}CKO | Stab-wound

n.s. **** ****

D

HRP | Control | Srf^{GFAP}CKO | Stab-wound

E

Color intensity (outside/inside)

Control | Srf^{GFAP}CKO | Stab-wound

n.s. **** ****
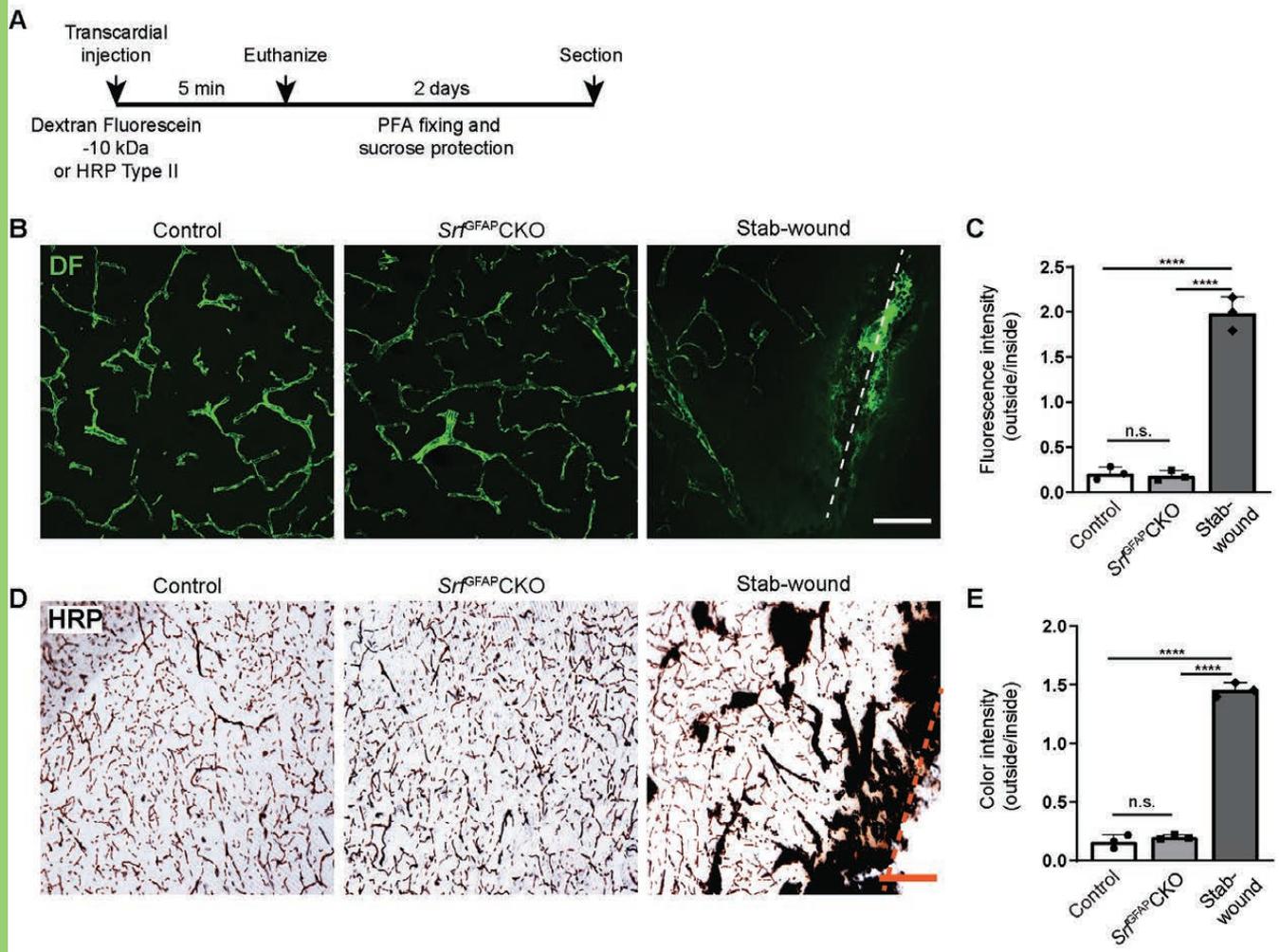
Figure 6

Figure 7

Figure 8

A

A2 astrocytic markers

Relative fold change

□ Control
■ *Srf*^GFAP^CKO

Cd109   Ptgs2   Clcf1   Cd14
  *   ns   *   *

A1 astrocytic markers

Relative fold change

Psmb8   H2T23   H2D1   Srgn
  **   ns   ns   ns

Pan reactive markers

Relative fold change

Serpina3n   Gfap
   **   ***

Neuroinflammatory genes

Relative fold change

Il1β   Ccl2/Mcp-1   TNFα
  *   **   ns

B

Control

Srf^GFAP^CKO

C

□ Control
■ *Srf*^GFAP^CKO

Average cell numbers/ROI

Cortex   Striatum
  ns   ns

Average cell numbers/mm²

CA1   CA3
 ns   ns
Hippocampus

D

□ Control
■ *Srf*^GFAP^CKO

Average cell numbers/ROI

Cortex   Striatum
  n.s   n.s

Average cell numbers/mm²

CA1   CA3
 n.s   n.s
Hippocampus