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3 **Connexin 43-mediated Neurovascular Interactions Regulate Neurogenesis in the**
4 **Adult Brain Subventricular Zone**
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26 **Abstract (150 words)**

27 The subventricular zone (SVZ) is the largest neural stem cell (NSC) niche in the
28 adult brain; herein, the blood-brain barrier is leaky, allowing direct interactions between
29 NSCs and endothelial cells (ECs). Mechanisms by which direct NSC-EC interactions in
30 the SVZ control NSC behavior are unclear. We found Cx43 to be highly expressed by
31 both NSCs and ECs in the SVZ, and its deletion in either cell type leads to increased NSC
32 proliferation and neuroblast generation, suggesting that Cx43-mediated NSC-EC
33 interactions maintain NSC quiescence. This is further supported by in vitro studies
34 showing co-culture with ECs decreases NSC proliferation and increases their expression
35 of genes associated with quiescence in a Cx43-dependent manner. Cx43 mediates these
36 effects in a channel-independent manner involving its cytoplasmic tail and ERK activation.
37 Such insights further advance our understanding of NSC regulation in vivo and may
38 inform NSC maintenance ex vivo for stem cell therapies for neurodegenerative disorders.

39

40 Introduction

41 In the adult mouse and human brains, neural stem cells (NSCs) reside in two
42 germinal niches, the dentate gyrus of the hippocampus, or subgranular zone (SGZ), and
43 the subventricular zone (SVZ) of the lateral ventricles, which is the largest NSC niche. In
44 mice, both niches allow the replenishment of new neurons throughout life; the SGZ is
45 involved in hippocampal neurogenesis while the SVZ enables olfactory bulb (OB)
46 neurogenesis. The SVZ is a polarized niche, where neurogenesis is initiated when glial
47 fibrillary acidic protein (GFAP⁺) quiescent NSCs are activated and become epidermal
48 growth factor receptor (EGFR⁺) NSCs that give rise to mammalian achaete-shute
49 homolog 1 (Mash-1⁺) transit-amplifying progenitor cells (TACs)¹⁻⁴. On the ventricular side
50 of the murine SVZ, quiescent NSCs protrude apical processes with short primary cilia that
51 contact the ependymal cell layer lining the ventricles and cerebrospinal fluid within the
52 ventricles^{1,5,6}. On the parenchymal side, quiescent NSCs project long basal end-feet that
53 make direct contact with blood vessels in the niche⁷.

54 In the SVZ, TACs assemble into small clusters and differentiate into chains of
55 doublecortin (DCX⁺) neuroblasts⁸; TACs are fast-dividing while neuroblasts proliferate ten
56 times slower^{2,6,9,10}. The chains of neuroblasts, along with some clusters of TACs, migrate
57 tangentially from the SVZ through a restricted pathway called the rostral migratory stream
58 (RMS) toward their destination in the OB^{2,11}. Once in the OB, neuroblasts exit the RMS
59 and migrate radially into the granular and periglomerular layers to differentiate into
60 interneurons^{9,12-14}. In the adult SVZ, NSCs predominantly undergo symmetric,
61 differentiative and consuming divisions to generate TACs, which gradually depletes the
62 pool of quiescent NSCs over time^{15,16}. Therefore, it is important to understand the
63 signaling cues between NSCs and the other SVZ niche cells, especially vascular
64 endothelial cells (ECs), to tightly regulate NSC activation, differentiation, migration, and
65 neurogenesis.

66 It is well-established that SVZ microvascular ECs are fundamental to the niche and
67 support NSC self-renewal, maintenance, proliferation, differentiation, and migration of
68 neural progenitor cells^{17,18}. Several studies have highlighted the role of paracrine
69 signaling between vascular ECs and NSCs in the SVZ via EC-secreted factors such as,

70 vascular endothelial growth factor (VEGF)-A and -C^{19,20}, betacelluline²¹, pigment
71 epithelium-derived factor (PEDF)²² and placental growth factor (PIGF) type 2²³ that
72 regulate NSC behavior in the SVZ. Moreover, in the SVZ microvasculature, and unlike
73 other areas of the brain, the ECs that form the blood vessels lack pericyte and astrocyte
74 coverage, enabling direct contact between NSCs and ECs in this niche^{18, 24,25}. This allows
75 for physical interactions between NSCs and ECs to enable juxtacrine signaling via
76 membrane-bound proteins and their associated ligands to enable Notch and Ephrin
77 signaling^{25,26}. Intercellular junctions, such as gap junctions, also form between NSCs and
78 ECs²⁷. Gap junctions enable intercellular signaling via the transfer of ions, metabolites,
79 soluble factors and molecules of $\leq 1\text{KDa}$. In the developing mouse brain, gap junction
80 proteins connexin 43 (Cx43) and Cx26 are differentially expressed and play important
81 roles in the regulation of neurogenesis^{28,29}. Gap junctions comprised of Cx43 are known
82 to form between NSCs and ECs in the mouse SVZ and RMS at various postnatal stages²⁷.
83 However, the role of EC- or NSC- expressed Cx43 in the regulation of adult SVZ
84 neurogenesis is not known and the focus of this study.

85 Herein, using an in-vitro transwell co-culture system, we showed that Cx43-
86 mediated interactions between NSCs and ECs decrease NSC proliferation and increase
87 their survival. Furthermore, in adult mice in which *Gja1*, which encodes for Cx43 protein,
88 has been conditionally deleted in either NSCs or ECs for 1 week, there is an initial
89 increase in NSC activation and the generation of DCX⁺ neuroblasts. However, after 4
90 weeks deletion, there is a significant reduction of quiescent NSCs in the SVZ and
91 increased neurogenesis in the OB. Finally, we show that deletion of *Gja1* in ECs impairs
92 the repopulation of the SVZ niche upon infusion of the antimitotic drug cytosine- β -
93 arabinofuranoside (Ara-C). Collectively, these studies suggest that Cx43-mediated
94 interactions between NSCs and ECs maintain NSC quiescence to regulate neurogenesis
95 in the adult SVZ. Such insights can be applied to the development of ex vivo biomimetic
96 engineered SVZ niches to further study NSC regulation and to potentially use them to
97 treat neurovascular disorders such as stroke.

98 **Results**

99 ***NSC-EC co-culture decreases NSC proliferation in a Cx43-dependent manner.***

100 To investigate the effects of ECs on NSC behavior, we used a 2D transwell system
101 where ECs were co-cultured in contact with NSCs allowing for direct cell-to-cell
102 interactions (**Figure 1a and 1b**). In this co-culture model, NSCs were seeded in the
103 absence of EGF and FGF (the growth factors that support NSC survival and growth) to
104 specifically assess the role of ECs in the regulation of NSC behavior. We observed that
105 ECs promote NSC survival, while NSC proliferation measured via EdU incorporation is
106 significantly decreased (**Figure 1c and 1d**). Bulk mRNA sequencing (RNAseq) of NSCs
107 in co-culture with ECs revealed that genes associated with NSC quiescence, such as
108 *Gfap*, *Sox9* and *Prom1*, are upregulated, while genes associated with NSC activation,
109 such as *Egfr* and *Ccne1*, are downregulated in comparison with NSCs cultured alone
110 (**Figure 1e**). Gene Ontology (GO) analysis revealed that genes associated with
111 neurogenesis are downregulated in NSCs when co-cultured with ECs (**Figure 1f**).
112 Additionally, quantitative (q) PCR analysis performed on NSCs co-cultured with ECs
113 showed increased mRNA levels of *Gfap*, *Nestin* and *Glast*, genes associated with NSC
114 quiescence while expression of *Egfr*, *Mash1*, and *Cyclin E*, genes associated with NSC
115 activation, are decreased (**Figure 1g**).

116 The RNAseq analysis also revealed the upregulation of potential regulators of
117 NSC-EC interactions (**Figure 1e**), including *Gja1* (also referred to as *Cx43*), which
118 encodes the gap junction protein Cx43. This was of interest to us, as we previously found
119 Cx43 mediates interactions between ECs and vascular mural cells³⁰. Thus, we used
120 qPCR analysis to confirm that *Cx43* expression is increased in NSCs when co-cultured
121 with ECs (**Figure 1h**). To determine whether Cx43 plays a role in mediating NSC-EC
122 interactions that lead to changes in NSC gene expression, we used siRNA to suppress
123 *Cx43* expression in ECs and then co-cultured them with NSCs. This resulted in
124 significantly decreased *Gfap* and *Glast* mRNA levels, and significantly upregulated *Egfr*,
125 *Mash1* and *Cyclin E* mRNA levels (**Figure 1i**). Conversely, when we silenced *Cx43* in
126 NSCs and then co-cultured them with ECs, we did not observe changes in NSC gene

127 expression (**Extended Figure 1a-e**). Collectively, these results suggest that ECs may
128 enhance NSC survival and quiescence via Cx43.

129

130 ***ECs and NSCs in the adult brain SVZ highly express Cx43.***

131 In our RNAseq studies, we found that *Gja1/Cx43* was the only gap junction gene
132 significantly upregulated in NSCs when co-cultured with ECs (**Figure 1e**); however, both
133 cell types have been shown to express other Cx proteins^{31,32}. Thus, we measured the
134 expression of different Cx proteins in ECs and NSCs *in vivo* in the adult mouse brain SVZ.
135 Using immunohistochemistry and antibodies against different Cx proteins and CD31,
136 which is expressed by all ECs, we found a low percentage of ECs expressing Cx26 and
137 Cx31, while a high percentage of ECs express Cx43 (**Figure 2a**). To evaluate the
138 expression of Cx proteins in NSCs in the murine SVZ, we first labeled the NSCs using a
139 BrdU-labeling protocol in which label-retaining cells (LRC) represent quiescent NSCs³³.
140 We found that a percentage of SVZ LRC-NSCs express multiple Cx proteins, and a higher
141 percentage of LRC-NSCs express Cx43 (**Figure 2b**), similar to ECs. To determine
142 whether Cx43 is present between NSCs and ECs in the SVZ *in vivo*, we performed
143 immunohistochemistry and high-resolution confocal imaging of SVZ coronal sections. We
144 found punctate-like Cx43 expression between CD31⁺ ECs and GFAP⁺SOX2⁺ NSCs, as
145 well as between SOX2⁺ cells of the ependymal layer. (**Figure 2c**).

146 To determine whether NSC- and/or EC-expressed Cx43 plays a role in the
147 regulation of NSCs in the adult brain SVZ, we used *Gja1*^{fl/fl} mice (hereafter referred to
148 as *Cx43*^{fl/fl} mice) and an inducible loss-of-function genetic approach. To selectively delete
149 *Cx43* in ECs, we crossed *Cx43*^{fl/fl} with *Cdh5Cre*^{iERT2} to generate *Cx43EC*^{iKO} mice, and to
150 selectively delete *Cx43* in NSCs, we crossed *Cx43*^{fl/fl} mice with *GlastCre*^{iERT2} to generate
151 *Cx43Glast*^{iKO} mice. To validate Cre-mediated recombination in ECs and NSCs in these
152 models, we crossed both *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice with *ROSA*^{mT/mG} mice in
153 which, upon tamoxifen (Tx) injection, cell-membrane localized tdTomato converts to GFP
154 in Cre recombinase-expressing cells. We performed recombination and genetic deletion
155 in 6-week-old (adult) mice with Tx injection, as shown in the timeline in **Figure 2d**. As

156 expected, we observed GFP⁺ ECs in the SVZ of *Cx43EC^{iKO};ROSA^{mT/mG}* mice and GFP⁺
157 NSCs in the SVZ of *Cx43Glast^{iKO};ROSA^{mT/mG}* (**Figure 2e**).

158 To further confirm the loss of Cx43 expression in our mouse models, we also
159 performed immunostaining with antibodies against CD31 to label ECs and antibodies
160 against SOX2 and GFAP to co-label NSCs. In the SVZ of *Cx43EC^{iKO}* mice, we found loss
161 of Cx43 expression in the CD31⁺ ECs and maintenance of Cx43 expression in ependymal
162 cells and NSCs. In the SVZ of *Cx43Glast^{iKO}* mice, we found Cx43 expression was lost in
163 GFAP⁺SOX2⁺ NSCs while ECs and ependymal cells retained expression (**Figure 2f**).
164 Cx43 deletion efficiency in ECs of *Cx43EC^{iKO}* and NSCs of *Cx43Glast^{iKO}* was confirmed
165 via qPCR analysis of primary SVZ cells dissociated and FACS-isolated into the EC
166 (CD31⁺CD45⁻Glast⁻) and NSC (Glast⁺CD45⁻CD31⁻) fractions (**Extended Figure 2**). Our
167 studies showed that in *Cx43EC^{iKO}* mice, ECs lost ~90% of Cx43 expression, which was
168 maintained in NSCs. Conversely, in *Cx43Glast^{iKO}* mice, Cx43 expression was suppressed
169 by ~75% in NSCs and maintained in ECs (**Figure 2g**). These results demonstrate
170 effective deletion of Cx43 in SVZ ECs and NSCs in Tx-induced *Cx43EC^{iKO}* and
171 *Cx43Glast^{iKO}* mice, respectively.

172

173 ***Cx43 deletion in ECs or NSCs depletes quiescent NSCs in the adult SVZ.***

174 We next analyzed the consequences of short- (1-week post-Tx) and long-term (4
175 weeks post-Tx) deletion of Cx43 in *Cx43EC^{iKO}* and *Cx43Glast^{iKO}* mice, compared to
176 control *Cx43^{fl/fl}*, on the number of quiescent and activated NSCs and neuroblasts in the
177 adult SVZ (**Figure 3a and 3b**). The identification of quiescent NSCs is still a challenge
178 due to lack of specific markers. In our study, we used combined expression of the
179 astrocytic marker GFAP and the neural stem and progenitor cell marker SOX2 to identify
180 NSCs^{25,34,35}. Since SOX2 and GFAP are expressed by both quiescent and activated
181 NSCs¹⁸, we injected *Cx43EC^{iKO}* and *Cx43Glast^{iKO}* mice, at 1 week and 4 weeks post-Tx,
182 with EdU 24 hr prior to sacrifice to measure quiescent NSCs (EdU⁻) and activated NSCs
183 (EdU⁺).

184 At 1-week post-Cx43 deletion, we observed a decrease in the number of
185 GFAP⁺SOX2⁺EdU⁻ quiescent NSCs in both *Cx43EC^{iKO}* and *Cx43Glast^{iKO}* SVZ (**Figure 3c**

186 **and 3i)** and further reduction at 4 weeks post-Tx (**Figure 3f and 3i**). Additionally, the
187 absence of GFAP⁺ cells co-expressing S100 β in the striatum of the mutant SVZs shows
188 that the decrease in quiescent NSCs observed at 4 weeks post-Cx43 deletion is not due
189 to abnormal astrogliosis (**Extended Figure 3a**). We also observed significantly increased
190 EGFR⁺SOX2⁺EdU⁺ activated NSCs in both mutant SVZs at 1-week post-Tx (**Figure 3d**
191 **and 3j**); however, at 4 weeks post-Tx, the number of activated NSCs was significantly
192 decreased (**Figure 3g and 3j**). Neuroblasts, identified via expression of DCX, were
193 significantly increased in both *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} SVZ at 1-week post-Cx43
194 deletion (**Figure 3e and 3k**), while their number was significantly reduced in both mutants
195 at 4 weeks post-Tx (**Figure 3h and 3k**). Furthermore, we used *Cx43Glast*^{iKO};ROSA^{mT/mG}
196 mice to perform lineage-tracing at 1 week post-Cx43 deletion. We found DCX⁺
197 neuroblasts (**Extended Figure 3b**, white arrow heads) localized within the recombinant
198 GFP⁺ population, supporting that the neuroblasts generated in the *Cx43Glast*^{iKO} SVZ
199 (**Figure 3e and 3k**) were derived from *Cx43*-deficient NSCs.

200 Elsewhere in the brain, with exception of the SVZ, vascular ECs interact with
201 astrocytes and pericytes via Cx43, which promotes their blood-brain-barrier (BBB)
202 function³⁶⁻³⁸. Thus, we sought to determine whether deletion of *Cx43* in vascular ECs or
203 Glast-expressing astroglial cells compromised BBB integrity and caused vascular
204 leakage. To assess vascular permeability, we injected *Cx43*^{f/f}, *Cx43EC*^{iKO} and
205 *Cx43Glast*^{iKO} mice with 2% Evans blue 24 hr prior to sacrifice, as depicted in **Extended**
206 **Figure 4a**, and evaluated vascular leakage in brain and liver tissues. As expected, we
207 did not observe vascular leakage in the control brains; there was also no leakage in the
208 mutant brains (**Extended Figure 4b**). In contrast, in liver tissues that lack barrier function,
209 leakage of Evans blue dye from the vasculature was evident throughout control and
210 mutant tissues (**Extended Figure 4c**). In addition, using Vascupaint green perfusion, we
211 did not observe any differences in *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} brain microvasculature
212 morphology at 4 weeks post-Tx, compared to *Cx43*^{f/f} controls. Thus, deletion of *Cx43* in
213 vascular ECs or astroglial cells does not appear to compromise BBB integrity in the brain.
214 We also measured brain and OB areas and found no differences between *Cx43*^{f/f} control

215 mice and *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice after short- or long-term deletion of *Cx43*
216 (**Extended Figure 5**).

217

218 ***Cx43 deletion in ECs or NSCs increases neuroblast generation in the RMS and***
219 ***neurogenesis in the OB.***

220 Since neuroblasts generated in the SVZ migrate toward the OB via the RMS, we
221 next assessed whether increased neuroblast generation in the *Cx43EC*^{iKO} and
222 *Cx43Glast*^{iKO} SVZs at 1-week post-Tx leads to increased neuroblasts in the RMS (**Figure**
223 **4a**). On brain sagittal sections immunostained with anti-DCX, we analyzed the anterior
224 portion of the RMS recognized by its elbow-shaped morphology (**Figure 4b and 4c**). In
225 this region, we found significantly increased DCX⁺ neuroblasts in *Cx43EC*^{iKO} and
226 *Cx43Glast*^{iKO} mice, compared to *Cx43*^{fl/fl} controls (**Figure 4d and 4e**). We next examined
227 whether the observed increase in neuroblasts in *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} RMS at 1-
228 week post-Tx affects neurogenesis in the OB. To do so, we used a LRC protocol (**Figure**
229 **4f**), in which 6-week-old adult mice received 3 consecutive EdU injections on the first 3
230 days of a 5-day Tx-induction. At 4 weeks post-Tx, mice were analyzed via immunostaining
231 with anti-EdU and anti-NeuN, to label newborn neurons^{2,39}. Interestingly, we observed a
232 significantly increased number of LRC/NeuN⁺ interneurons in the granule cell layer (GCL)
233 of both *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} OB compared to *Cx43*^{fl/fl} controls (**Figure 4g-4i**).

234 To determine whether neurons in the *Cx43Glast*^{iKO} RMS were derived from SVZ
235 NSCs, we used *Cx43Glast*^{iKO}; *ROSA*^{mT/mG} mice to perform lineage-tracing studies
236 (**Extended Figure 6a**). We found LRC/NeuN⁺ cells (**Extended Figure 6b**, white arrows)
237 localized within the recombinant GFP⁺ population, supporting that the newborn olfactory
238 neurons generated in the *Cx43Glast*^{iKO} SVZ (**Figure 3e and 3k**) were derived from *Cx43*-
239 deficient NSCs. Importantly, the increase in newborn neurons in *Cx43EC*^{iKO} and
240 *Cx43Glast*^{iKO} OB was not a consequence of neuronal death, as we did not observe
241 changes in apoptosis levels in *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} OB measured via immuno-
242 staining for cleaved CASPASE-3 (**Extended Figure 6c-e**). Collectively, these results
243 suggest that the short-term deletion of *Cx43* in ECs or NSCs leads to decreased
244 quiescent NSCs and increased activated NSCs in the SVZ, as well as increased

245 neuroblast generation in the SVZ and RMS. After long-term deletion of EC- or NSC-
246 expressed Cx43, the quiescent NSC pool in the SVZ is further depleted, while activated
247 NSCs and neuroblasts are exhausted from the SVZ, and neurogenesis is increased in
248 the OB. Thus, both EC- and NSC-expressed Cx43 contributes to the regulation of adult
249 SVZ neurogenesis.

250

251 ***EC-expressed Cx43 is necessary for SVZ niche repopulation.***

252 Our studies suggested that NSC quiescence and maintenance in the SVZ niche is
253 regulated by ECs in a Cx43-dependent manner. Thus, we tested whether lack of EC-
254 expressed Cx43 would impair NSC-mediated SVZ niche repopulation after depletion of
255 all SVZ proliferating cells (activated NSCs, TACs and neuroblasts) via anti-mitotic drug
256 Ara-C (cytosine-β-D-arabinoside). At 1-week post-Tx injections in *Cx43EC^{iKO}* and *Cx43^{fl/fl}*
257 control mice, Ara-C (2%) or saline (control) was infused directly in one of the two lateral
258 ventricles (ipsilateral) via a cannula connected to a subcutaneously implanted mini-
259 osmotic pump for 6 consecutive days (**Figure 5a**). EdU was administered to label actively
260 proliferating cells 24 hr prior to sacrifice. The intracerebroventricular injection coordinates
261 were verified via injection of 1% Fast Green dye, as previously described⁴⁰ (**Extended**
262 **Figure 7a**).

263 We first confirmed that Ara-C treatment depleted all EdU⁺ proliferating cells
264 (**Figure 5b and 5c**), as well as DCX⁺ neuroblasts (**Figure 5d and 5e**), in the SVZ.
265 Importantly, we observed increased DCX⁺ neuroblasts in control, saline-infused
266 *Cx43EC^{iKO}* SVZ compared to *Cx43^{fl/fl}* (**Figure 5d and 5e**). These results are consistent
267 with our previous studies (**Figure 3e and 3k**) where we demonstrated that short-term
268 deletion of Cx43 in *Cx43EC^{iKO}* leads to increased SVZ neuroblasts. Subsequently, we
269 analyzed the number of DCX⁺ neuroblasts in the SVZ 6 days after Ara-C withdrawal
270 (**Figure 5f**, chase period). DCX⁺ neuroblasts were significantly reduced in *Cx43EC^{iKO}*
271 SVZ, compared to *Cx43^{fl/fl}* (**Figure 5g and 5h**). Thus, absence of Cx43 in ECs impairs
272 the re-population of the SVZ niche post-Ara-C treatment, supporting that Cx43 expression
273 in ECs contributes to the maintenance of the quiescent NSCs pool in the SVZ and
274 prevents their premature activation and depletion.

275 ***Cx43 cytoplasmic tail mediates EC-induced NSC quiescence in an ERK-dependent***
276 ***manner.***

277 We next investigated the mechanism(s) by which Cx43 regulates NSC-EC
278 interactions to maintain NSC quiescence. Cx43 can function in a channel-dependent or
279 channel-independent manner; thus, we created lentiviral constructs to express Cx43
280 mutant proteins to perform structure-function studies to determine how Cx43 mediates
281 NSC-EC interactions. To investigate channel-dependent functions, we generated a Cx43
282 channel-dead mutant (*Cx43T154A*) that allows gap junction channel formation but blocks
283 Cx43 channel activity⁴¹. To investigate channel-independent functions, we generated
284 mutant Cx43 that lacks its cytoplasmic tail (*Cx43CTΔ258*)⁴², which can mediate
285 intracellular signaling, independent of channel formation⁴³. ECs and NSCs were treated
286 with siCx43 to suppress endogenous Cx43 expression, and then transduced with lentiviral
287 constructs to express the mutant Cx43 proteins. Expression of the Cx43 cytoplasmic tail
288 truncated mutant (*Cx43CTΔ258*, 1 µg), lead to a significant downregulation of genes
289 associated with NSC quiescence (*Gfap*, *Nestin* and *Glast*), while expression of the Cx43
290 dead-channel mutant (*Cx43T154A*, 0.5 µg) had no effect on NSC gene expression
291 (**Figure 6a, Extended Figure 8a and 8b**). Additionally, when we treated both NSC and
292 EC with ⁴³gap 26 peptide (100 nM), which blocks Cx43 channel activity, we did not
293 observe any changes in NSC gene expression associated with either quiescence or
294 activation (**Figure 6b**). Thus, it appears that EC-mediated NSC quiescence is regulated
295 by Cx43 in a channel-independent manner. We assessed whether overexpression of
296 *Cx43* in NSCs alone was sufficient to suppress NSC proliferation, as measured via EdU
297 incorporation, and found that Cx43 overexpression in NSC does not affect their
298 proliferation (**Extended Figure 8c-g**).

299 It is known that the Cx43 cytoplasmic tail is involved in the activation of
300 downstream effectors involved in ERK signaling^{43,44} and, interestingly, we observed
301 increased ERK activation in NSCs when co-cultured with ECs and the latter is abrogated
302 in NSCs co-cultured with ECs silenced for Cx43 (**Figure 6c and 6d**). Finally, inhibition of
303 ERK signaling with U0126 (10 µM) in NSCs co-cultured with ECs abolished the effect of
304 ECs on NSC expression of genes associated with quiescence (**Figure 6e**). Collectively,

305 these results suggest that EC maintenance of NSC quiescence is mediated by the Cx43
306 cytoplasmic tail, via ERK activation in NSCs.

307 **Discussion**

308 NSCs in the adult brain SVZ reside in a vascularized niche, which is known to
309 regulate their proliferation, migration, and differentiation via niche cell-derived secreted
310 factors^{21,22,45-49}. It is also well established that quiescent NSC basal end-feet are in direct
311 contact with vascular ECs allowing neuro-vascular interactions⁷. Past studies showed that
312 the expression of Cx43 in neural progenitor cells maintains their survival and proliferative
313 state^{27,50-53}. However, the role of Cx43-mediated neuro-vascular interactions in the adult
314 SVZ in the regulation of NSC behavior has not been investigated.

315 In this study, we first used an in vitro EC-NSC co-culture system that allows direct
316 cell-to-cell contact^{54,55} to demonstrate that ECs decrease NSC proliferation and increase
317 their expression of genes associated with quiescence in a Cx43-dependent manner. We
318 then used transgenic mice to show that deletion of Cx43 in ECs or NSCs in vivo leads to
319 increased NSC proliferation and neuroblast generation in the SVZ, as well as increased
320 neurogenesis in the OB. Our observations are consistent with previously published
321 studies showing that the vascular niche maintains quiescent NSCs and promotes their
322 survival^{25,56}, and provide insight into the underlying mechanisms of regulation.

323 Interestingly, our in vivo results show the same outcome whether Cx43 is deleted
324 in ECs or NSCs while, in vitro, only EC-expressed Cx43 mediates effects on NSC
325 proliferation and survival. There are many differences between the two systems that can
326 account for this finding, including lack of other SVZ components in NSC-EC co-cultures
327 that may be important for this regulation. In addition, in the in vitro studies, NSCs were
328 silenced for Cx43 prior to co-culture with ECs while in vivo NSCs and ECs were in contact
329 prior to Cx43 deletion in either ECs or NSCs. Thus, there are likely more complex
330 interactions between ECs and NSCs, and perhaps other cell types, in the SVZ niche that
331 depend on EC- and NSC-expressed Cx43.

332 The evaluation of the short- vs. long-term deletion of Cx43 in either ECs or NSCs
333 showed that the SVZ quiescent NSC pool is gradually depleted over time and leads to
334 increased neuroblasts in the RMS and ultimately increased newborn neurons in the OB.
335 Consistent with this, when quiescent NSCs in the Cx43EC^{iKO} SVZ were triggered towards
336 activation to replenish the depleted niche post-AraC infusion, the number of neuroblasts

337 generated in *Cx43EC^{iKO}* was significantly less than controls. This finding is consistent
338 with the idea that deletion of *Cx43* in ECs activates quiescent NSCs in the adult SVZ
339 which leads to a gradual exhaustion of the NSC pool over time; thus, the NSCs in the
340 *Cx43EC^{iKO}* SVZ cannot replenish the niche to the same extent as control littermates.
341 Whether the increased newborn neurons in the OB leads to functional neurons that fully
342 integrate the olfactory neuronal circuits and modify the olfaction behavior of these *Cx43*-
343 deficient animals is not yet known.

344 Using mutant *Cx43* proteins, we demonstrated that *Cx43* mediates EC regulation
345 of NSCs in a channel-independent manner that involves its cytoplasmic tail and ERK
346 activation. However, further mechanistic studies are needed to determine how *Cx43* is
347 regulating ERK activation in NSCs co-cultured with ECs. For example, whether the
348 phosphorylation of *Cx43* on a particular serine residue of the cytoplasmic tail is
349 responsible for ERK activation remains to be addressed; however, the phosphorylation
350 serine sites 255, 279 and/or 282 of the *Cx43* cytoplasmic region were previously linked
351 with ERK activity⁵⁷. Also, it has been shown that activated ERK can bind to *Cx43*,
352 suggesting that *Cx43* may play a direct role in ERK regulation⁵⁸. We found that EC-NSC
353 co-culture increases *Cx43* expression in NSCs; however, over-expression of *Cx43* in
354 NSCs does not mimic the effects of EC-expressed *Cx43* on NSC behavior or gene
355 expression. Thus, although it is possible that, in NSCs, ERK signaling via the *Cx43*
356 cytoplasmic tail enables the activation of downstream effectors that promote NSC
357 quiescence, the regulatory mechanisms are likely to be more complex.

358 In summary, we used both in vitro and in vivo approaches to gain insight into the
359 role of *Cx43* in the regulation of NSCs in the adult SVZ. We show that EC- and NSC-
360 expressed *Cx43* are required to maintain NSC quiescence. Further mechanistic studies
361 are needed to determine exactly how *Cx43* is regulating NSC behavior in the adult SVZ,
362 and our in vitro studies suggest that this occurs in a channel-independent manner. Such
363 insights can be applied to the bioengineering of a NSC niche ex vivo that better mimics
364 its in vivo environment, to enable sustained NSC viability and functional properties for
365 stem cell therapies for neurological disorders.

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370

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372 G.M., performed experiments. S.M. and M.R.H. maintained mouse lines and performed
373 mouse genotyping. B.R.A generated Cx43 lentiviral constructs. N.G., G.G. and K.K.H
374 designed experiments, analyzed data and wrote the manuscript. A.E., J.L.T., C.F.C
375 contributed to experimental design and manuscript editing.

376

377 **Material and Methods**

378 **Animals.** *Cdh5Cre*^{ERT2}1Rha (also referred to as *VE-CadherinCre*^{ERT2})^{59,60} and
379 *GLASTCre*^{ERT2} (*Slc1a3Cre*^{ERT2})⁶¹ were gifts from Drs. Ralf Adams and Jean Léon
380 Thomas labs, respectively. *Gja1*^{fl/fl} (also referred to as *Cx43*^{fl/fl}) and *ROSA*^{mT/mG}
381 mice were commercially purchased. For genetic loss-of-function studies, *Cdh5Cre*^{ERT2}
382 and *GLASTCre*^{ERT2} animals were crossed to mice carrying a loxP-flanked *Gja1* gene
383 (*Cx43*^{fl/fl}) to create *Cx43EC*^{iKO} and *Cx43Glast*^{iKO}, respectively. Mice were maintained
384 under standard pathogen-free conditions. All animal protocols and procedures were
385 reviewed and approved by the University of Virginia Animal Care and Use Committee
386 (protocol #4277) and complied with all ethical regulations. To induce Cre activity in
387 *Cx43EC*^{iKO} and *Cx43Glast*^{iKO}, 6-week-old adult mice received intraperitoneal (i.p.)
388 injections of Tamoxifen (Tx, 2mg/day) for 5 consecutive days, which resulted in ~90%
389 and ~75% *Gja1* deletion in ECs of *Cx43EC*^{iKO} and NSCs of *Cx43Glast*^{iKO} respectively, as
390 assessed by qPCR (Figure 3E). Tx-injected *Cx43*^{fl/fl} littermates were used as controls.
391 Mice of both sexes were used to minimize gender-related biased results and analyzed at
392 1 week-post final Tx injections for short-term deletion studies and 4 week-post final Tx
393 injections for long-term deletion studies. For Ara-C studies, mice were analyzed at 2- and
394 3-week-post final Tx injection. To label actively proliferating progenitors in the SVZ, mice
395 received an i.p. injection of EdU (50mg/kg) 24 hr prior to sacrifice. We used label retention
396 to identify quiescent NSCs that are slow-cycling cells, also known as label retaining cells
397 (LRC) that retain BrdU or EdU for extended periods due to their relatively long cycling
398 times^{18,62,63}. To do so, mice received 5 consecutive injections of BrdU or 3 consecutive
399 EdU injections on the first 3 days of the 5 days of Tx injections, mice were analyzed 4-
400 weeks post-Tx.

401

402 **Mouse genotyping.** Ear sample DNA was lysed using the hotshot method.
403 Samples were lysed in alkaline lysis reagent (pH 12) containing 25mM NaOH and 0.2mM
404 EDTA for 1 hr at 90 °C. Subsequently, samples were neutralized using 40mM Tris-HCl.
405 PCR was performed in PCR-ready tubes (Bioneer Inc. K-2016) containing 1µL of DNA

406 sample and 12.5 mM primers for a final reaction volume of 20 μ L adjusted with RNase-
407 free H₂O. PCR genotyping primers sequences are documented in **Table 1**.
408

<i>GlastCre</i> ^{ERT2}	Forward sequence (5'-3'): GAGGCACTTGGCTAGGCTCTGAGGA Reverse sequence 1 (5'-3'): GAGGAGATCCTGACCGATCAGTTGG Reverse sequence 2 (5'-3'): GGTGTACGGTCAGTAAATTGGACAT
<i>Cdh5Cre</i> ^{ERT2}	Forward sequence (5'-3'): AATCTCCCACCGTCAGTACG Reverse sequence (5'-3'): CGTTTCTGAGCATACTGGGA
<i>MTMG</i>	MTMGC (Common) (5'-3'): CTCTGCTGCCTCCTGGCTTCT MTMGMR (mutant reverse) (5'-3'): TCAATGGCGGGGGTCGTT MTMGWR (wild-type reverse) (5'-3'): CGAGGC CGGATCAC AAGCAATA
<i>Gja1</i>	Forward sequence (5'-3'): ACAGCGGTTGAGTCAGCTTG Reverse sequence (5'-3'): GAGAGATGGGAAGGACTTGT

409 **Table 1: PCR genotyping primers sequences**
410

411 **Tissue collection, subventricular zone (SVZ), rostral migratory stream (RMS) and**
412 **OB immunohistochemistry and imaging.** Mice were sacrificed with a lethal dose of
413 ketamine (80mg/kg body weight)/xylazine (8mg/kg body weight). Mice received trans-
414 cardiac infusion of 10mL sterile PBS supplemented with 2mM EDTA and 10U/mL heparin
415 followed by 10mL 3.7% formaldehyde. Brain tissues were post-fixed with 3.7%
416 formaldehyde at 4°C overnight. Post-fixation, brains were washed 3 times during 10 min

417 in PBS 1X. SVZ coronal sections, sagittal sections of the RMS and coronal sections of
418 the OB were collected in a 24-well plate in a sequential manner and subsequently
419 subjected to a free-floating slice immunostaining protocol. All sections were cut at 50 μ m
420 with a Leica vibratome (VT1000S). Briefly, slices were permeabilized with 0.5% Triton X-
421 100 for 30 min at room temperature (RT). If EdU staining was required, we proceeded
422 with EdU staining post-permeabilization following the manufacturer's protocol (Click-iTTM
423 – ThermoFischer C10337). Sections were then blocked with 10% donkey serum in PBS
424 1X supplemented with 0.3% Triton X-100 (blocking buffer) for 1 hr at RT. After blocking,
425 sections were incubated with appropriate primary antibodies (listed in **Table 2**) diluted in
426 blocking buffer overnight at 4°C, on a low-speed rocking plate. Samples were washed
427 three times with PBS 1X supplemented with 0.1% Triton X-100 (PBST) then incubated
428 with respective conjugated secondary antibodies for 1 hr at RT. After 3 washes with
429 PBST, Hoechst (4 μ M) was added during 30 min for nucleus counterstaining. SVZ images
430 were acquired with an inverted Leica SP8 DMi8 high-resolution confocal microscope
431 equipped with adaptive deconvolution (LIGHTNING[®], Leica) using 63x or 20x objectives.
432 A series of 3 to 5 SVZ slices from each animal taken from the same rostro-caudal area,
433 judged by the shape of the lateral ventricles, of the corpus callosum and the anterior
434 commissure were imaged. We localized the RMS attached to the OB between a sagittal
435 depth of 3.2-3.35mm from the lateral side of the brain toward the midline. For OB analysis,
436 the whole OB was cut coronally. Images were post-analyzed using ImageJ and
437 Photoshop (Adobe) software.

438

Antibody/catalogue number	Species	Dilution
Connexin 43 (Abcam ab11370)	Rabbit	1:1000
SOX2 (ebioscience 14-9811-82)	Rat	1:100
EGFR (Millipore 06-847)	Rabbit	1:75
Doublecortin (Abcam ab18723)	Rabbit	1:200
Doublecortin (AVES lab DCX)	Chicken	1:200
GFAP (DAKO Z0334)	Rabbit	1:400
GFAP (AVES lab GFAP)	Chicken	1:400
PECAM1 (CD31) (Millipore MAB1398Z)	Hamster	1:100
NeuN (Abcam ab104225)	Rabbit	1:500
S100 β (DAKO Z0311)	Rabbit	1:300
CASPASE-3 (Cell signaling 9661S)	Rabbit	1:200

439 **Table 2: Primary antibodies used for SVZ immunostaining**

440 **Evans blue permeability assay.** To assess changes in vascular permeability, mice
441 received an i.p. injection of 2% Evans blue and were sacrificed 24 hr later. Prior to brain
442 and liver harvesting, mice were infused with PBS 1X supplemented with 2mM EDTA and
443 10U/mL heparin to wash out any traces of blood and maintain vessel integrity. Brains
444 were post-fixed in 3.7% formaldehyde overnight and imaged using a dissecting
445 microscope equipped with a digital camera (Leica).

446

447 **Vacupaint™ Silicone Rubber injection.** To assess changes in the brain
448 microvacuature, mice were injected with 4 ml of Vascupaint (green) following the
449 manufacturer's protocol (ediLumine™). Post-vascupaint injection, brains were harvested
450 and post-fixed in 3.7% formaldehyde overnight. Brains were then dehydrated with 30%,
451 60% and 100% methanol successively for 24h each. Post-dehydration, brains were
452 clarified with 50:50 of Benzyl Benzoate:Benzyl alcohol for 48h and imaged using a Nikon
453 SMZ-745T Trinocular 4K Digital Stereo microscope.

454

455 **Ara-C infusions.** Cytosine- β -D-arabinofuranoside (Ara-C) infusions were performed
456 as previously published^{6,64}. Briefly, 2% Ara-C (Sigma) in 0.9% saline or saline alone was
457 infused directly in the lateral ventricle (ipsilateral side) of adult mice (8-week-old) via a
458 cannula (Alzet[®] brain infusion kit 3) implanted stereotactically in a 1mm burr hole drilled
459 on the surface of the brain at the following coordinates: 1.4 mm lateral and 0.5 mm rostral
460 to bregma. Intracerebroventricular injection coordinates were verified with the injection of
461 Fast Green dye 1% as previously described⁴⁰ (**Extended Figure 5**). The cannula was
462 connected to a subcutaneously implanted mini-osmotic pump (Alzet[®] model 1007D flow
463 rate 0.5 μ l/hr, 7 days). After 6 days of infusion, the pump was removed, and mice were
464 sacrificed at the indicated survivals. The success of the Ara-C infusion was evaluated by
465 immunostaining for the neuroblast marker, DCX and the proliferation marker EdU.

466

467 **FACS isolation of primary ECs and NSCs from mouse brain cortex and SVZ.**

468 Primary ECs and NSCs from mouse brain cortex and SVZ were FACS-isolated
469 following a detailed published protocol⁶⁵. Briefly, brain cortex and SVZ were harvested (5
470 brains at a time to maximize cell viability), minced into small pieces of \sim 1 x 1 mm in size.
471 Minced pieces are then subjected to enzymatic dissociation in collagenase/dispase
472 (Roche, 100mg/ml stock) solution for 30 min at 37°C in a hybridization oven with constant
473 rotation. Subsequently, the digested tissues were triturated in 2% FBS in PBS
474 supplemented with DNase (10 mg/ml stock solution) with a P1000 pipette (\sim 100x).
475 Afterward, debris and myelin were removed with Percoll, cells were pelleted by
476 centrifugation and resuspended in HBSS/BSA/glucose buffer for immunostaining.
477 Antibodies (**Table 3**) were added to the resuspended cells (1 μ l of antibody/10⁵ cells) and
478 incubated during 20 min on ice protected from light. Post-immunostaining cells were
479 washed with HBSS/BSA/glucose buffer and filtered through a 40 μ m cell strainer. Cells
480 isolated from the cortex were used as unstained, single-color and FMO controls (each
481 combination of all antibodies except one), as well as isotype controls to set up gating and
482 compensation strategy (**Extended Figure 3**). From SVZ CD45 $^+$ cells, ECs (CD31 $^+$ Glast $^+$
483 population) and NSCs (Glast $^+$ CD31 $^+$ population) were collected using a BD FACS Melody
484 cell sorter equipped with a 100 μ m nozzle in pre-filled FACS tubes with EC and NSC

485 media respectively to cushion the cells. Post-FACS processing, cells are pelleted, snap
486 frozen in liquid nitrogen and stored at -80 °C for RNA isolation.
487

Antibody/catalogue number	Isotype
Glast-FITC (Alomone AGC-021-F)	Rabbit IgG
CD31-PE (BD Pharmingen™ 553373)	Rat IgG _{2a}
CD45-V450 (BD Pharmingen™ 560501)	Rat IgG _{2b}

488 **Table 3: Antibodies used for FACS**

489 **RNA isolation.** RNA from primary ECs and NSCs, as well as bEnd.3 and ANS4-GFP
490 cells, were purified using RNeasy Plus micro kit (Qiagen). 1 µg of RNA was reverse
491 transcribed using high-capacity cDNA Reverse transcription kit (Applied Biosystems™).
492 Quantitative PCR was performed on 15 ng cDNA using PowerUp SYBR Green Master
493 Mix (Applied Biosystems™) and the corresponding primers (**Table 4**). The data were first
494 normalized to actin level in each sample, and the relative expression levels of different
495 genes were calculated by the comparative Ct method⁶⁶.

Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Actin</i>	AGAGGGAAATCGTGCCTGAC	CAATAGTGATGACCTGGCCGT
<i>Connexin 43</i>	ACAGCGGTTGAGTCAGCTTG	GAGAGATGGGAAAGGACTTGT
<i>Cyclin E</i>	TTCTGCAGCGTCATCCTCT	TTCTGCAGCGTCATCCTCT
<i>EGFR</i>	GCTTGCAACGGTTCTCTCTC	CCACTGCCATTGAACGTACCCAG
<i>GFAP</i>	ACCATTCTGTACAGACTTTCTCC	AGTCTTACACGATGTTCTCTT
<i>Glast</i>	AAGCATCACAGCCACGGCCG	GTTGGAGGCGGTCCAGAAACC
<i>Mash1</i>	ATGCAGCTACTGTCCAAACG	AACAGTAAGGGGTGGGTGTG
<i>Nestin</i>	CTGCAGGCCACTGAAAGTT	GACCCTGCTTCTCCTGCTC

496 **Table 4: Mouse primers used for quantitative PCR**

497
498 **siRNA transfection.** Cx43 siRNA (Smartpool siRNA Cx43, L-051694-00-0005) and
499 the negative control/SiScramble (Non-targeting pool siRNA, D-001810-10-05) were
500 purchased from Dharmacon. We transfected bEnd.3 cells when 70% confluent with 60nM
501 siRNA per six-well plate. Experimentally, lipofectamine RNAimax (Invitrogen) is mixed
502 with opti-MEM media (Gibco) and incubated at room temperature for 5 min (mix A). 60nM

503 of SiCx43 premixed Opti-MEM is then added to mix A and incubated for 15 min (mix B).
504 Mix B is then added to bEnd.3 cells DMEM media (ATCC) without penicillin/streptomycin.
505 The same protocol was used to transfect ANS4-GFP cells with 40nM of SiCx43. 60nM
506 and 40nM of SiScramble (control) were used to transfect bEnd.3 and ANS4-GFP cells,
507 respectively, for control experiments. Cells were used for experiments 72 hr post-
508 transfection.

509 **Gja1 overexpression in ANS4-GFP.** *Gja1* overexpression in ANS4-GFP was
510 performed using a pcDNA3.1⁺ /C-(K)DYK vector containing *mus musculus Gja1* (Cx43)
511 cDNA (GenScript NM_010288.3). Transfection was performed according to the
512 manufacturer's experimental protocol in 6-well plate. To assess proliferation, EdU (5 μ M)
513 was added to the culture media 24 hr post-transfection. At 48 hr post-transfection, NSC-
514 GFP were collected for qPCR and western blot analysis to assess transgene expression
515 or fixed for immunofluorescence studies to assess Cx43 expression and EdU uptake.

516 **Generation of Cx43 mutants.** Human *GJA1* (gene for Cx43) full length (FL) construct,
517 Cx43 cytoplasmic tail truncated at amino acid 258 (*Cx43 Δ CT258*) and dead channel Cx43
518 mutant (*Cx43T154A*, mutation that converts threonine 154 into alanine) were PCR-
519 amplified from pTRE-TIGHT-Cx43-eYFP (gifted from Robin Shaw; Addgene Plasmid
520 #31807) and cloned into the pcDNA3.1-HA (gifted from Oskar Laur; Addgene Plasmid
521 #128034). We inserted the PCR fragments at NheI/BamHI sites by infusion HD-cloning
522 (Takara Biosciences), to excise HA-tag from the plasmid, rendering the constructs
523 expressed as tag-less. Site-directed mutagenesis was achieved using Q5-Site-Directed
524 Mutagenesis Kit (NEB, USA), according to the manufacturer's protocol. Primers were
525 designed using NEB-Base changer software (**Table 5**). Plasmids were sequenced and
526 confirmed by Eurofin Sanger Sequencing Services, USA.

527

528

Gene Name	Primers
Human <i>GJA1</i> full length (FL)	Forward primer 5'-3': ACCCAAGCTGGCTAGCATGGGTGACTGGAGCGCC Reverse primer 5'-3': CTGGACTAGTGGATCCCTAGATCTCCAGGTATCAGGCC
Human <i>Cx43ΔCT258</i>	Forward primer 5'-3': ACCCAAGCTGGCTAGCATGGGTGACTGGAGCGCC Reverse primer 5'-3': CTGGACTAGTGGATCCTTATTTGGCAGGGCTCAGCG
Human <i>Cx43T154A</i>	Forward primer 5'-3': GGGGTTGCTGCGAGCCTACATCATCAGTATCC Reverse primer 5'-3': GGATACTGATGATGTAGGCTCGCAGCAACCCC

529 **Table 5: Human *Cx43*full length (FL), *Cx43ΔCT258*and *Cx43T154A* primers sequences.**

530

531 **Cell culture.** ANS4-GFP cells were gifted from Dr. Steve M. Pollard (University of
532 Edinburgh). They were cultured as previously described⁶⁷⁻⁶⁹. bEnd.3 cells were
533 purchased from (ATCC CRL-2299™) and cultured in Dulbecco's Modified Eagle's
534 Medium (DMEM) (ATCC 30-2002) supplemented with 10% FBS and 1%
535 penicillin/streptomycin. ANS4-GFP cells were used up to passage (P) 30 and bEnd.3 up
536 to P16.

537

538 **Transwell co-culture of bEnd.3 and ANS4-GFP.** Transwell polycarbonate membranes
539 of a 12-well plate (Corning, catalogue #3401) were activated with media and bottom sides
540 were pre-coated with 0.1% gelatin for 1 hr at 37°C. The transwell inserts were then placed
541 with bottom side facing up in a 100mm tissue culture dish pre-filled with DPBS and bEnd.3
542 were then seeded. One hour later, inserts were flipped back in the wells of the 12-well
543 plate pre-filled with bEnd.3 media and top side was coated with laminin (10µg/ml
544 overnight at 37 °C). 24 hr-post-bEnd.3 seeding, the membrane was washed on both sides

545 with serum free media to remove any traces of serum that may cause differentiation of
546 ANS4-GFP cells. ANS4-GFP cells were then seeded on the top side of membrane in
547 ANS4 media without growth factors (EGF and FGF). bEnd.3 and ANS4 were co-cultured
548 at a 1:2 ratio in 100% ANS4-GFP media (supplemented with 5 μ M EdU when needed).
549 Membranes designated for ANS4-GFP monocultures were subjected to the same extra-
550 cellular matrix coating proteins as co-culture studies. ANS4-GFP were harvested after 48
551 hr of co-culture with trypsin for either qPCR analysis or fixed with 3.7% pre-warmed
552 formaldehyde for immunofluorescence studies. For *Gja1* knock-down studies: bEnd.3 cells
553 seeded on the bottom side of the membrane were transfected 24 hr before seeding ANS4-
554 GFP cells on the top side and subjected to 48 hr of co-culture prior to harvest. For studies
555 using siCx43 transfected ANS4-GFP: ANS4-GFP were transfected 24 hr prior to seeding
556 on the top side of the membrane and harvested after 48 hr of co-culture. For studies using
557 human *GJA1* mutants, ANS4-GFP and bEnd.3 were treated concomitantly with SiCx43
558 (see section siRNA transfection) and/or Cx43FL (1 μ g), Cx43T154A (0.5 μ g),
559 Cx43CT Δ 258 (1 μ g) for 6 hr. After SiCx43 and DNA transfection, cells were subjected to
560 a media change and harvested after 48 hr of co-culture. To block Cx43 channel activity,
561 after 24 hr of co-culture, ANS4-GFP and bEnd.3 cells were both treated with 100nM of
562 43 gap 26 peptide (VCYDKSFPISHVR) (Genscript, catalogue # RP20274), every 8 hr for
563 24 hr. To block ERK signaling in the co-culture system, ANS4-GFP cells were treated
564 with 10 μ M of U0126 (Cell signaling catalogue #9903S) for 24 hr. ANS4-GFP were
565 collected (6-8 wells are pooled per condition) either for qPCR or Western blot analysis
566 after 48 hr of co-culture.

567 **Immunofluorescence of transwell membranes.** Insert wells were washed with
568 DPBS supplemented with Ca $^{2+}$ and Mg $^{2+}$ allowing for cells to remain attached to the
569 membrane. Cells were fixed with pre-warmed 3.7% formaldehyde in DPBS supplement
570 with Ca $^{2+}$ and Mg $^{2+}$ for 15 min, then permeabilized with 0.5% Triton X-100 in PBS at room
571 temperature for 20 min prior to EdU staining according to the manufacturer's protocol
572 (Click-iTTM, Invitrogen). Cells were then blocked with 10% donkey serum and 0.1% Triton
573 X-100 (blocking buffer) for 1 hr at RT followed by incubation with primary antibodies,

574 chicken anti-GFP (1:500, Abcam ab13970) for NSCs and hamster anti-CD31 (1:500,
575 Millipore MAB1398Z) for bEnd.3 cells, in blocking buffer at 4°C overnight and
576 corresponding conjugated secondary antibodies for 1 hr at RT. Hoechst (5 µg/ml) was
577 used for nuclear counterstaining. Membranes were cut out from inserts and mounted with
578 DAKO mounting media (DAKO). Images were acquired with a DMi8 SP8 confocal
579 microscope at 4 different viewing fields and analyzed in Image J.

580

581 **Bulk RNA sequencing.** mRNA samples from ANS4-GFP and ANS4-GFP cells co-
582 cultured with bEnd.3 (for 72 hours) were isolated using RNeasy Plus Micro Kit (QIAGEN,
583 cat# 74034). Next-generation whole transcriptome Illumina sequencing (HiSeq4000) was
584 performed by the Yale Center for Genome Analysis. Fastq-files of raw reads were
585 generated with bcl2fastq2_v2.19.0 and then uploaded to the usegalaxy.org platform⁷⁰ for
586 quality control and trimming (Galaxy ToolShed v 1.0.2; FASTQ/A short-reads pre-
587 processing tools: http://hannonlab.cshl.edu/fastx_toolkit/)^{71,72}. Sequences were aligned to
588 the hg38genome using Kallisto⁷³ and aligned sequences were quantified with Sleuth⁷⁴.
589 Differential gene expression analysis was performed with the Sleuth package in R by the
590 Likelihood Ratio Test with regression of the experiment number to account for batch
591 effects.

592

593 **Western Blot.** Cells were lysed in RIPA buffer (Abcam, ab206996) and equal
594 amounts of proteins (quantified according to the Pierce™ BCA™ assay kit manufacture's
595 protocol, ThermoFischer, 23252) were separated on 4-15% gradient Criterion precast
596 gels (Bio-Rad 567-1084). Proteins were then transferred onto nitrocellulose membranes
597 (Bio-Rad). Western Blots were developed with chemiluminescence HRP substrate
598 (Radiance plus, Azure Biosystems AC2103) on a digital image analyzer, Azure Imager
599 c300.

600

601 **Label retaining cell (LRC) protocol.** The LRC protocol was carried out, as previously
602 described. Briefly, wild-type mice received BrdU (7.5mg/ml) i.p injections twice daily for 5
603 days and sacrificed 4 wk after the last injection. Quantification of connexin protein

604 colocalization with LRCs or ECs was performed on confocal z-stack images by a
605 computational approach using FARSIGHT for nuclear segmentation and MATLAB
606 programs as previously published in the lab³³.

607

608 **Data analysis and statistics.** All continuous variables were represented as mean \pm
609 SEM. The Mann-Whitney non-parametric test for unpaired samples was used to analyze
610 continuous variables between groups and p value ≤ 0.05 was considered statistically
611 significant. All analyses were performed using Prism 8.0 software (GraphPad).

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Figure 1**a**

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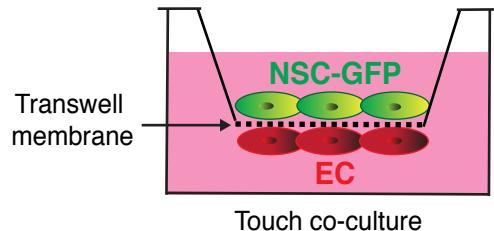
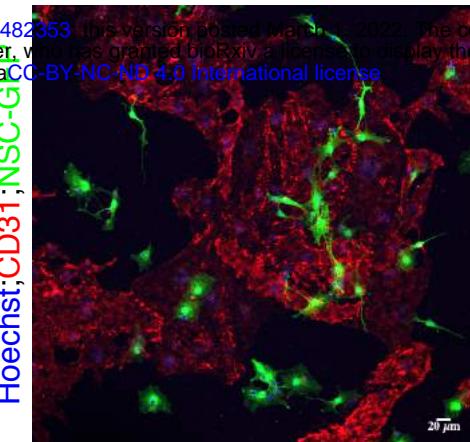
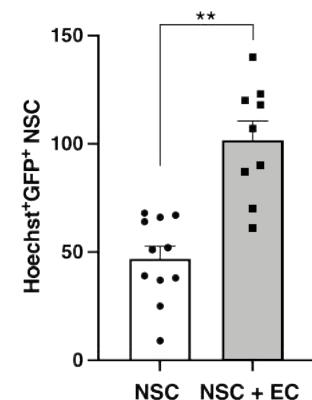
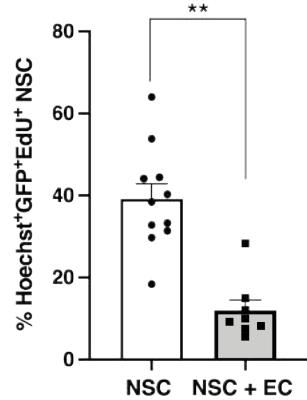
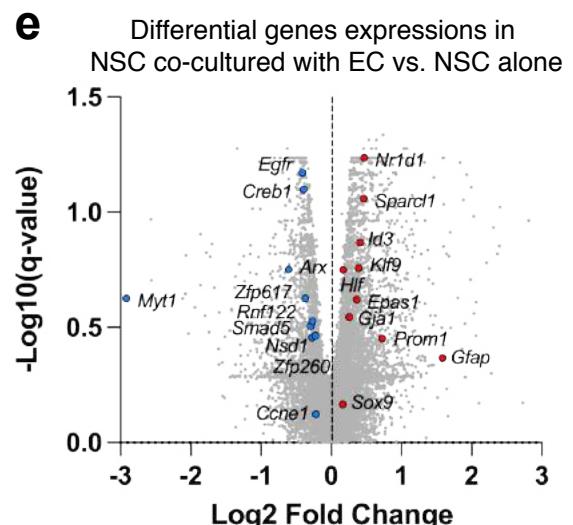
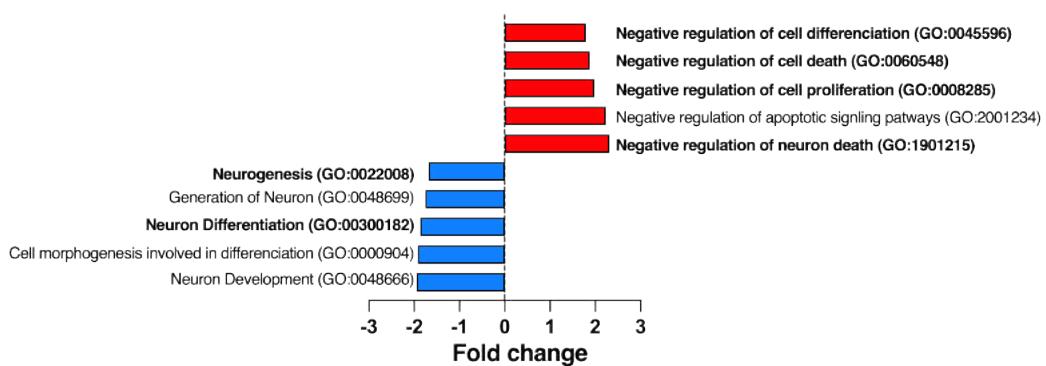
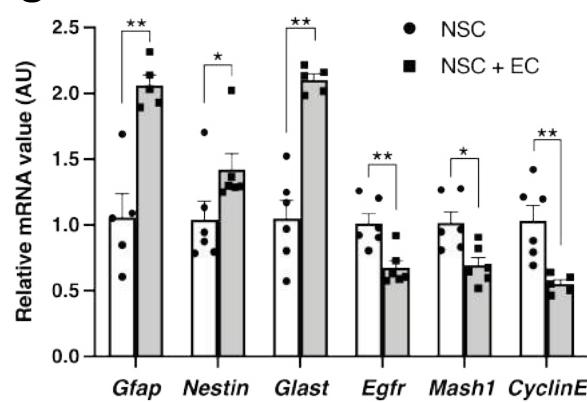
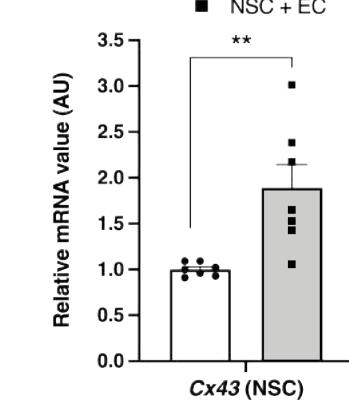
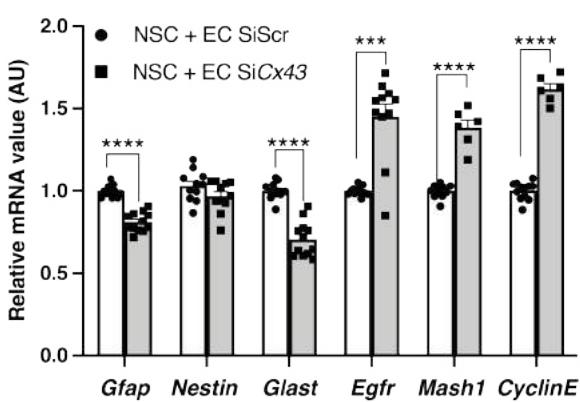
**b****c****d****e****f****g****h****i**

Figure 1: NSC-EC co-culture decreases NSC proliferation in a Cx43-dependent manner. (a) Schematic representation for transwell touch co-culture of vascular ECs (bottom side) with GFP⁺NSCs (top side). (b) Confocal image showing CD31⁺ EC (red) and GFP⁺ NSCs (green) co-cultured on the transwell membrane. (c) Quantification of total GFP⁺NSCs number and (d) percentage of GFP⁺NSCs with EdU uptake when cultured alone or with ECs respectively (n=3-4 biological replicates, 4 viewing fields/n). (e) Volcano plot of differential genes expressions in NSCs co-cultured with ECs compared to NSCs cultured alone. Red and blue dots indicated selected upregulated or downregulated genes respectively (n=3 biological replicates). (f) Gene ontology term analysis of selected gene family modified in NSCs co-cultured with ECs compared to NSCs cultured alone. (g) qPCR analysis of quiescent and activated genes in NSCs alone or NSCs co-cultured with ECs (n=3 biological replicates). (h) qPCR analysis of *Cx43* gene expression in NSCs alone or NSCs co-cultured with ECs (n=3 biological replicates). (i) qPCR analysis of quiescent and activated genes in NSCs co-cultured with ECs, where ECs are treated with control siRNA (siScr) or *Cx43* siRNA (Si*Cx43*) (n=3 different experiments). Data are mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

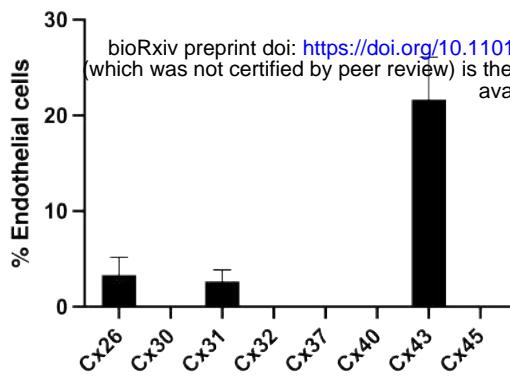
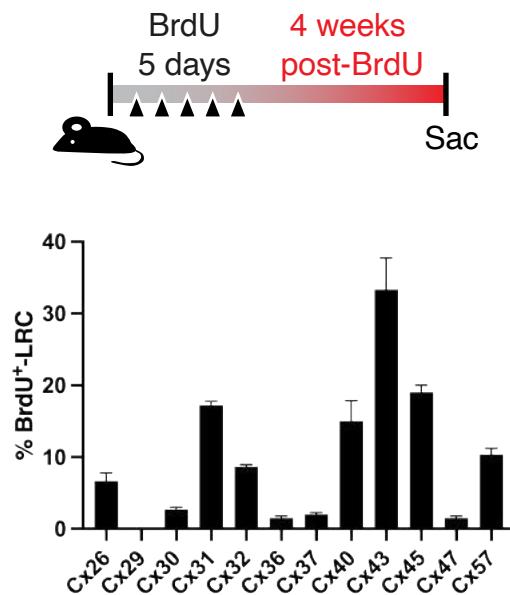
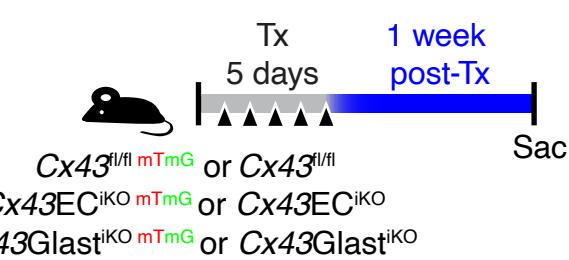
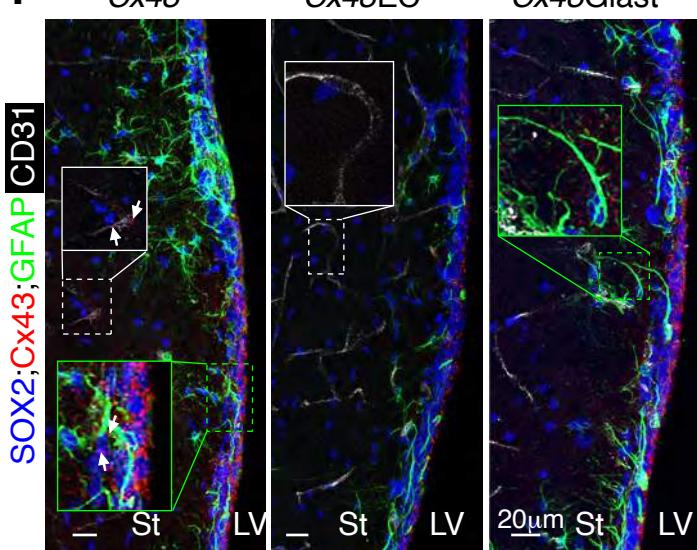
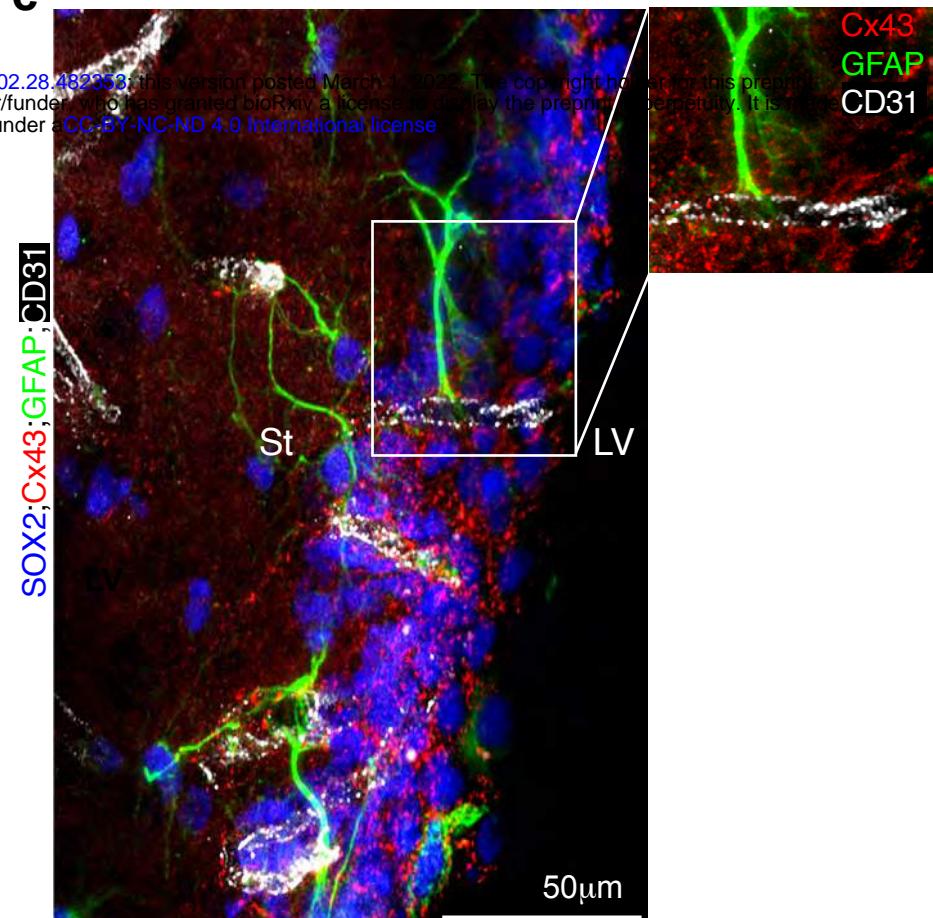
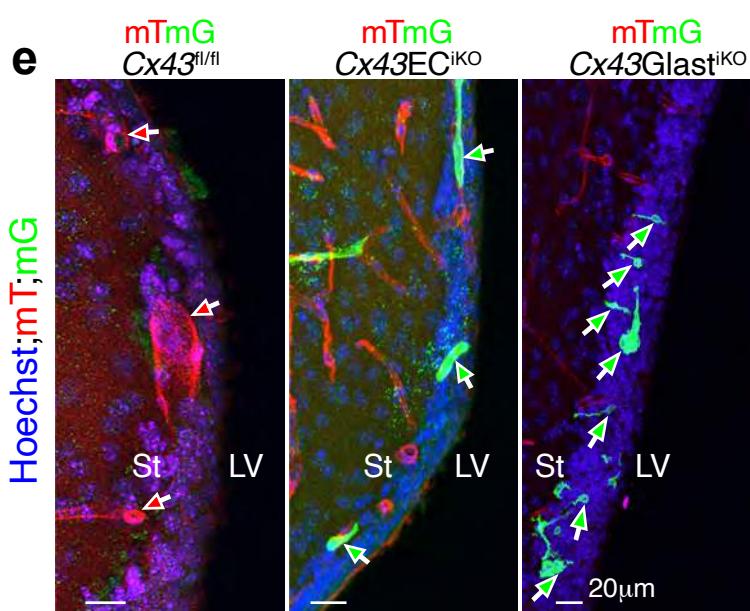
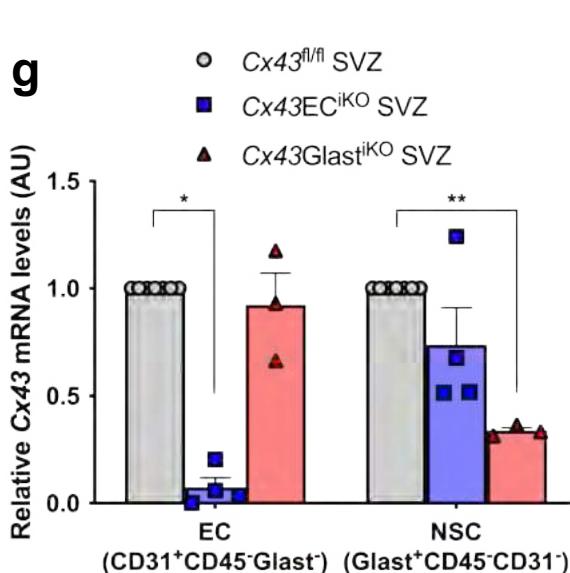
Figure 2**a****b****d****f****c****e****g**

Figure 2: ECs and NSCs in the adult brain SVZ highly express Cx43.

(a) and **(b)** Quantifications of the percentage of CD31⁺EC and BrdU⁺-LRC⁺ quiescent NSCs colocalizing with different Cx proteins respectively. **(c)** High resolution confocal image of a coronal section from a control mouse brain SVZ showing the end-feet of GFAP⁺SOX2⁺ NSCs (green and blue) contacting CD31⁺ endothelial cells via Cx43 punctate (inset). Scale bar is 50 μ m. **(d)** Timeline used to evaluate Cx43 deletion and recombination in the SVZ. **(e)** Representative confocal images of SVZ from Cx43EC^{iKO};ROSA^{mT/mG} and Cx43Glast^{iKO};ROSA^{mT/mG} mice. Note the presence of green recombinant ECs or NSCs (green arrows) and only red tomato cells in the Cx43^{fl/fl};ROSA^{mT/mG} SVZ (red arrows). **(f)** Representative confocal images of Cx43 immunostaining in the SVZ of Cx43^{fl/fl}, Cx43EC^{iKO} and Cx43Glast^{iKO} mice. Note that Cx43 punctate staining (white arrows) in ECs (white inset) and NSC (green inset) are specifically deleted in Cx43EC^{iKO} and Cx43Glast^{iKO} SVZ respectively. **(g)** Cx43 mRNA expression in ECs (CD31⁺CD45⁻Glast⁺ population) and NSCs (Glast⁺CD45⁻CD31⁻ population) of Cx43^{fl/fl}, Cx43EC^{iKO} and Cx43Glast^{iKO} SVZ (n=3-4 different experiments, 5 SVZs per pooled experiment). Scale bar is 20 μ m. St: Striatum; LV: Lateral ventricle. Data are mean \pm SEM. * p \leq 0.05, ** p \leq 0.01.

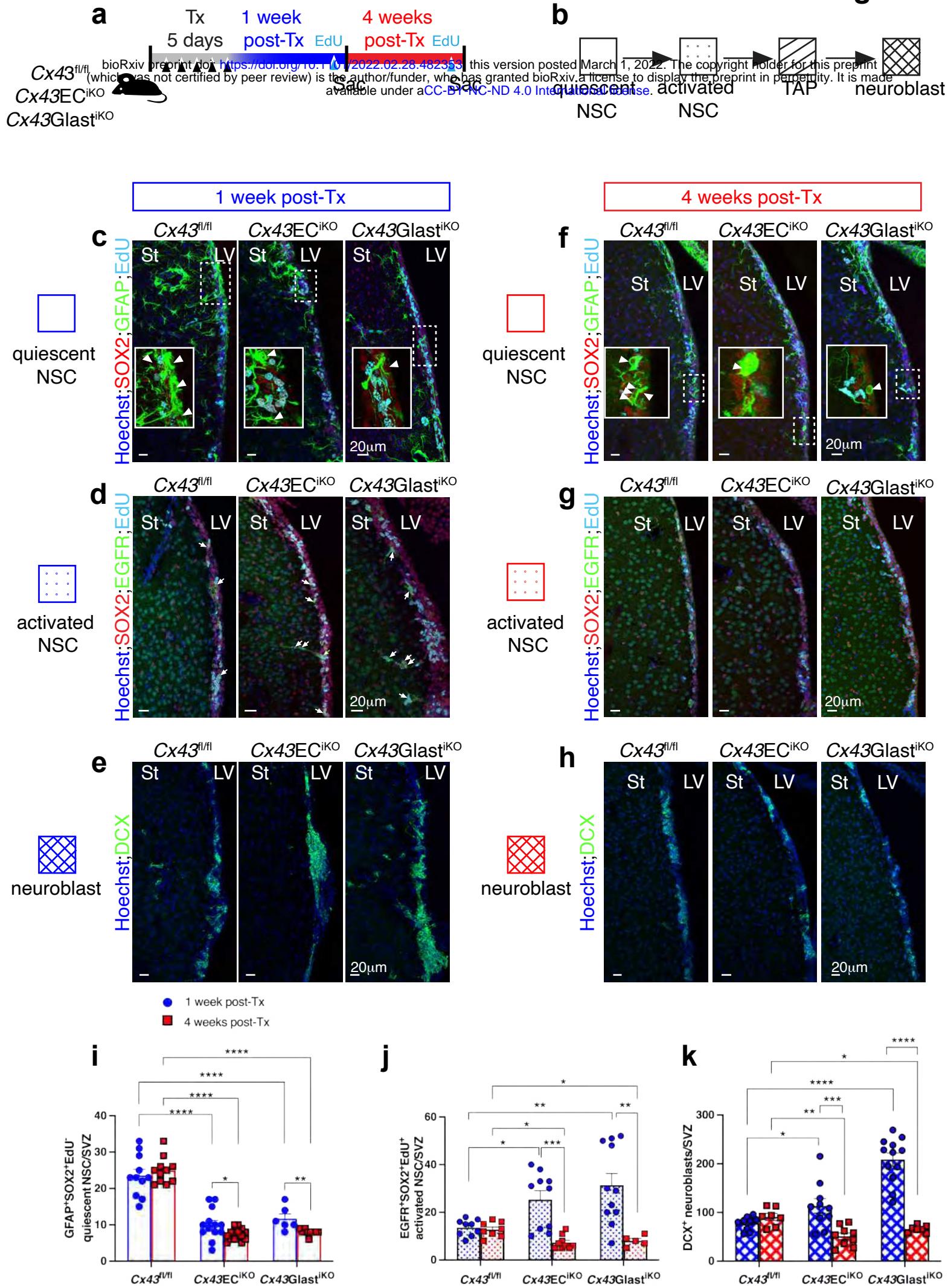
Figure 3

Figure 3: *Cx43 deletion in ECs or NSCs depletes quiescent NSCs in the adult SVZ.*

(a) Timeline used to evaluate the effects of short-term deletion (1-week post-Tx) vs long-term deletion (4-weeks post-Tx) of *Cx43* on the number of quiescent NSCs, activated NSCs and neuroblasts in the SVZ. **(b)** Schematic model of quiescent NSCs activation and differentiation in the SVZ. **(c)** to **(h)** Confocal images of SVZ coronal sections from *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice showing in **(c)** and **(f)** quiescent GFAP⁺SOX2⁺EdU⁻ NSC (arrowheads), **(d)** and **(g)** EGFR⁺SOX2⁺EdU⁺ activated NSCs (arrows in **d**); **(e)** and **(h)** DCX⁺ neuroblasts. **(i)** to **(k)** Quantifications of images shown in (c) to (h) (n=3-5 different animals per group). Scale bar is 20 μ m. St: Striatum; LV: Lateral ventricle. Data are mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.001$.

Figure 4

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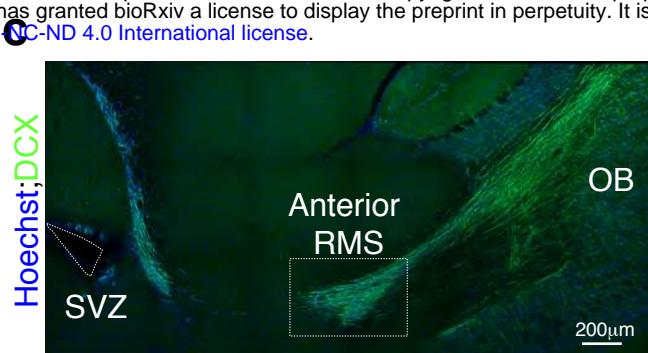
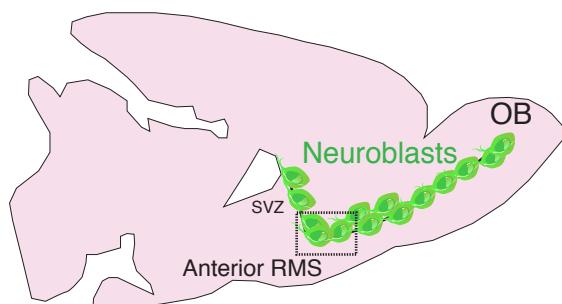
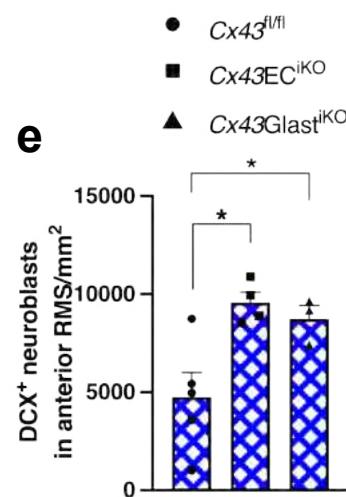
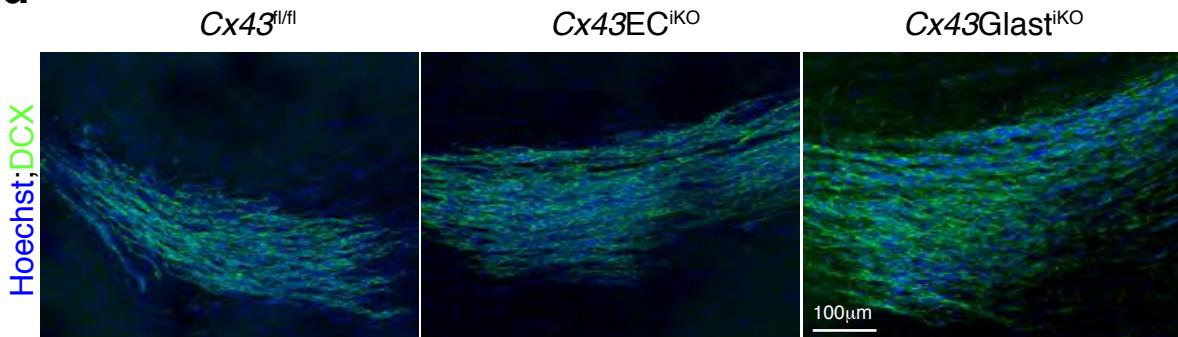
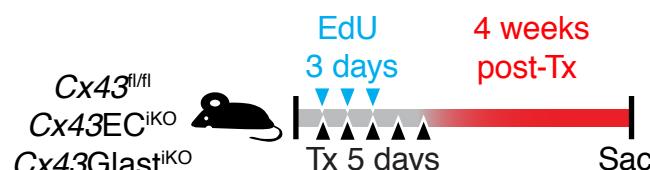
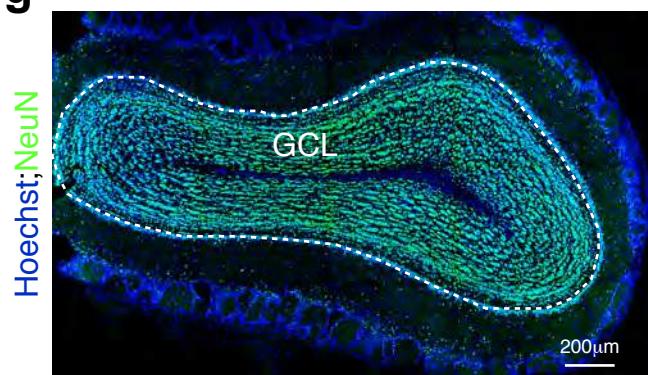
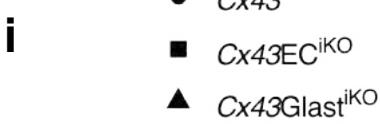
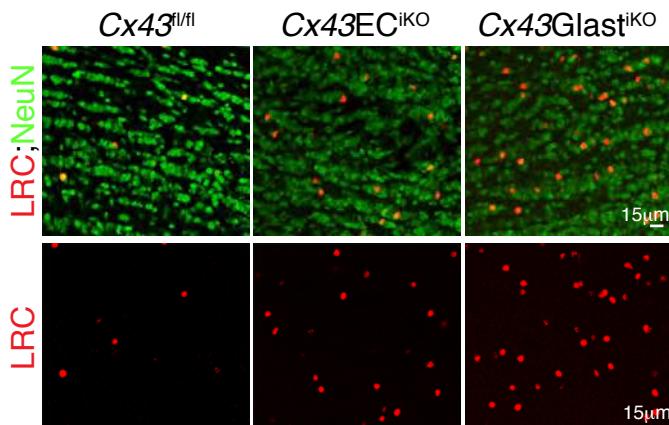
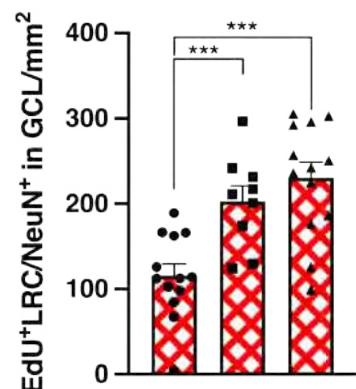
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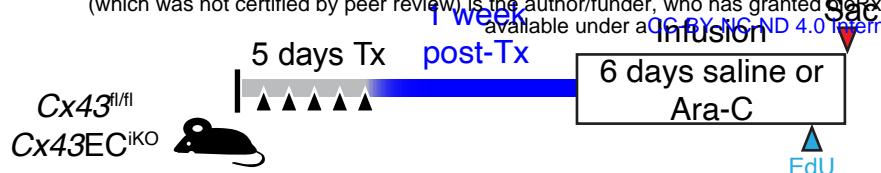
Figure 4: *Cx43 deletion in ECs or NSCs increases neuroblast generation in the RMS and neurogenesis in the OB.*

(a) Timeline used to evaluate the effects of short-term deletion of *Cx43* on neuroblasts in the RMS. **(b)** Schematic representation of a mouse brain coronal section (50 μm) showing the RMS. **(c)** a representative confocal image of DCX $^+$ neuroblasts in the RMS. Dashed boxes in **(b)** and **(c)** highlight the anterior RMS. **(d)** Representative confocal images of DCX $^+$ neuroblasts in the anterior RMS of *Cx43^{fl/fl}*, *Cx43EC^{iKO}* and *Cx43Glast^{iKO}* mice. **(e)** Quantifications of images shown in **(d)** (n=4 different images analyzed from 2 animals per group). **(f)** Timeline used to evaluate the effects of long-term deletion of *Cx43* on neurogenesis in the olfactory bulb. **(g)** Representative confocal image of NeuN $^+$ newborn neurons (in green) on a coronal section (50 μm) of the mouse olfactory bulb. The dashed white lines outline the granule cell layer (GCL). **(h)** Representative images of EdU $^+$ label retaining cells (LRC, red) and NeuN $^+$ newborn neurons (green) in GCL of *Cx43^{fl/fl}*, *Cx43EC^{iKO}* and *Cx43Glast^{iKO}* mice. **(i)** Quantifications of images shown in **(h)** (n=5-8 sections analyzed from 2 different animals per group). Scale bars are 200 μm in **(c)**, 100 μm in **(d)** and **(g)** and 15 μm in **(h)**. Data are mean \pm SEM. * $p \leq 0.05$, *** $p \leq 0.001$.

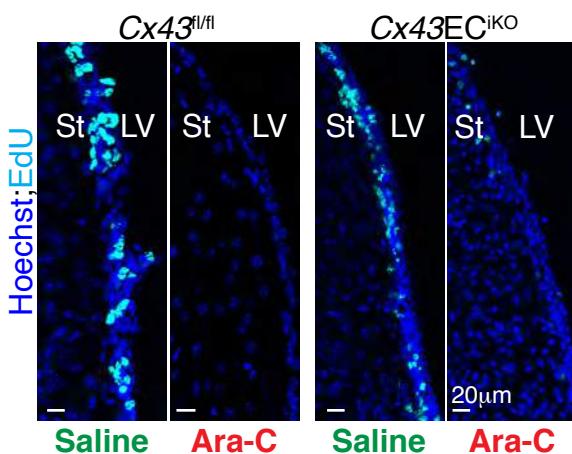
Figure 5

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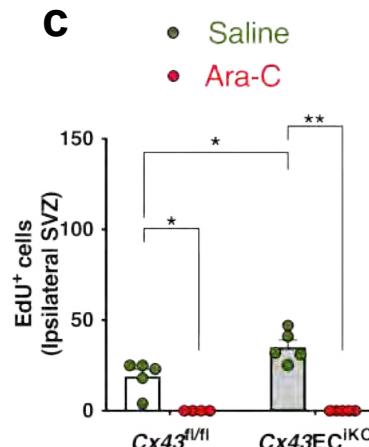
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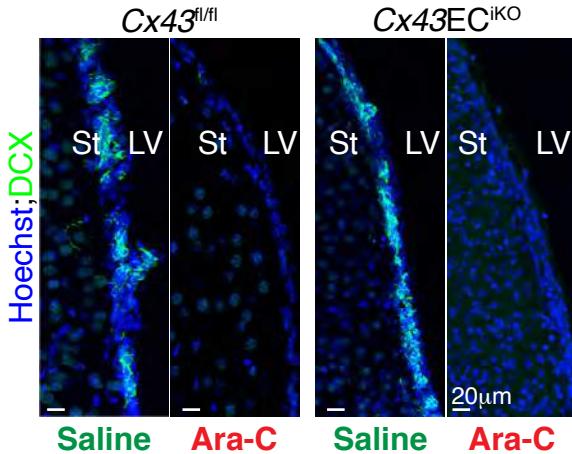
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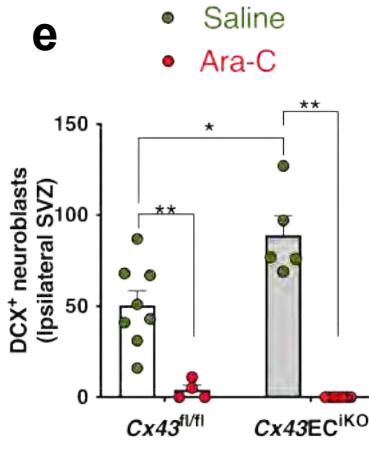
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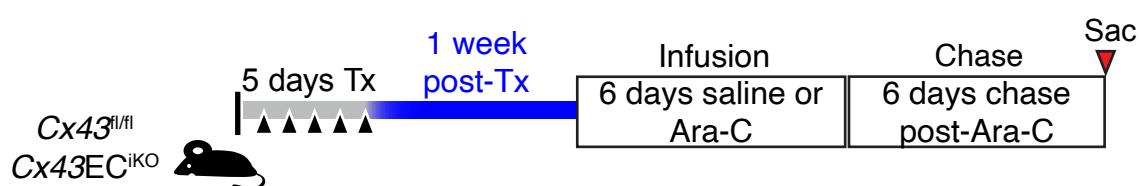
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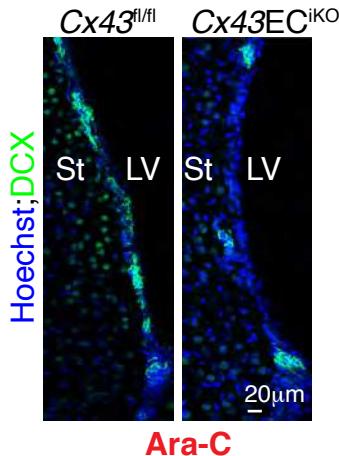
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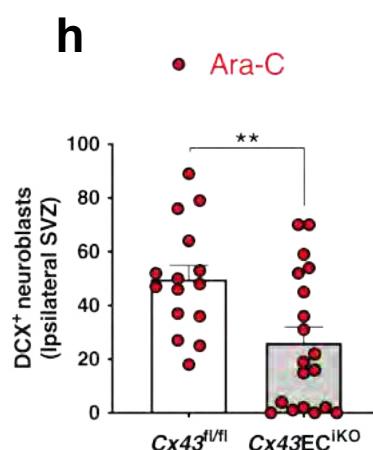


Figure 5: EC-expressed Cx43 is necessary for SVZ niche repopulation.

(a) Timeline used for saline or Ara-C infusions. (b) Representative confocal images showing EdU⁺ cells in SVZ of saline vs. Ara-C infused *Cx43*^{fl/fl} and *Cx43EC*^{iKO} mice after 6 days infusion. (c) Quantifications of images shown in (b) (n=2 different animals). (d) Representative confocal images showing DCX⁺ neuroblasts in SVZ of saline vs. Ara-C infused *Cx43*^{fl/fl} and *Cx43EC*^{iKO} mice. (e) Quantifications of images shown in (d) (n=2 biological replicates). (f) Timeline used for Ara-C infusion and chase period. (g) Representative confocal images showing DCX⁺ neuroblasts in SVZ of saline vs. Ara-C infused *Cx43*^{fl/fl} and *Cx43EC*^{iKO} mice after Ara-C infusion and chase period. (h) Quantifications of images shown in (g) (n=3 biological replicates). Scale bar is 20 μ m. St: Striatum; LV: Lateral ventricle. Data are mean \pm SEM. * p \leq 0.05, ** p \leq 0.01.

Figure 6

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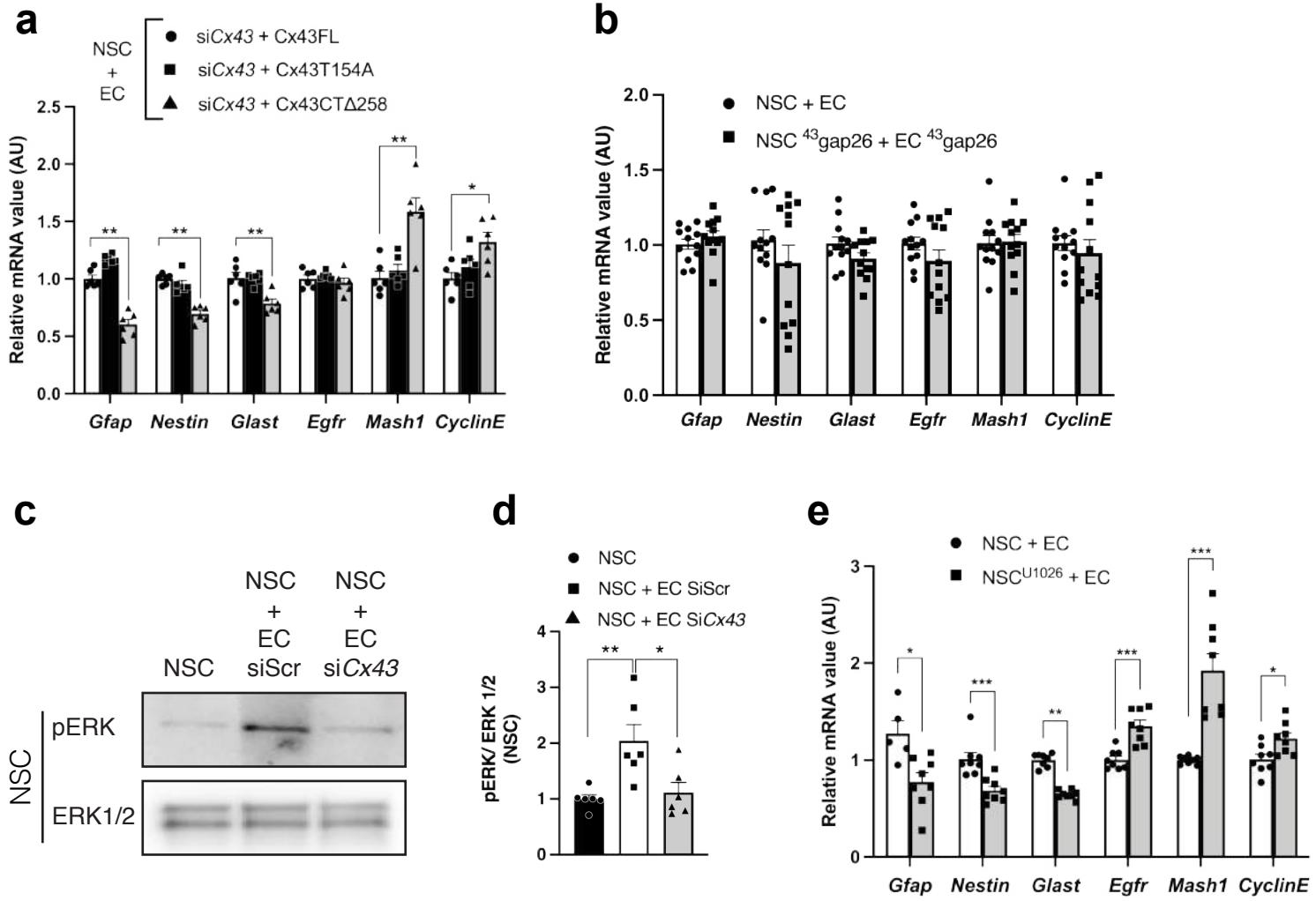


Figure 6: *Cx43 cytoplasmic tail mediates EC-induced NSC quiescence in an ERK-dependent manner.*

(a) qPCR analysis of quiescent and activated genes in NSCs co-cultured with ECs where both cell types are treated with SiCx43 followed by either the *Cx43T154A* mutant (Cx43 dead channel) or the *Cx43CTΔ258* mutant (cytoplasmic tail truncated) compared to NSCs, and ECs co-culture treated with SiCx43 followed by *Cx43* full length (FL) construct. (n=3 different experiments). **(b)** qPCR analysis of quiescent and activated NSCs co-cultured with ECs where both cell types are treated with ⁴³gap26 compared to NSCs and ECs co-culture (n=3 different experiments). **(c)** Western blot analysis of pERK and ERK protein levels in NSCs cultured alone, or co-cultured with ECs treated with control siRNA (SiScr) or *Cx43* siRNA (SiCx43) and quantifications are shown in **(d)** (n=6 different experiments). **(e)** qPCR analysis of quiescent and activated NSCs co-cultured with ECs where NSCs is treated with the ERK signaling inhibitor U0126 compared to NSCs and ECs co-culture (n=2 different experiments). Data are mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. **** $p \leq 0.0001$.

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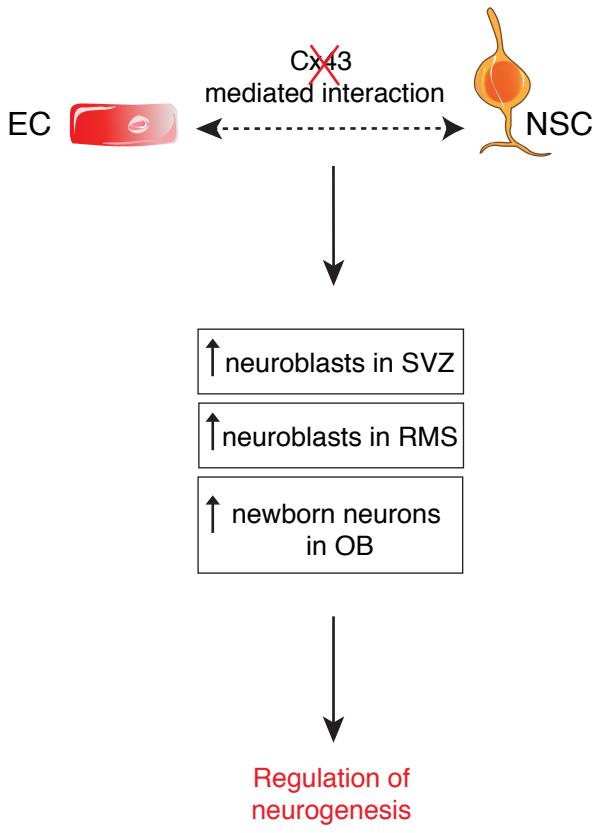
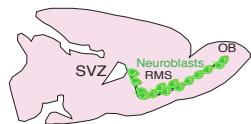
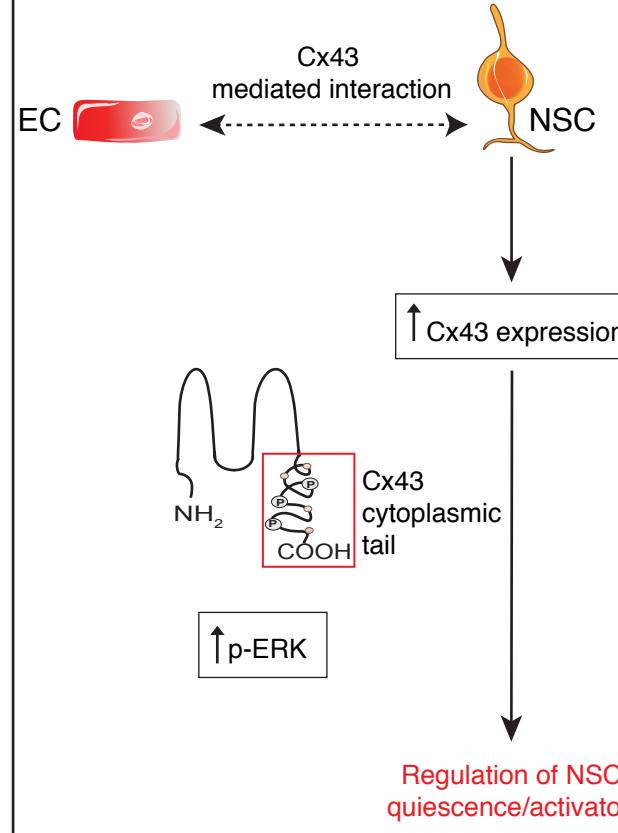
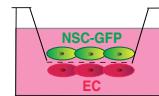
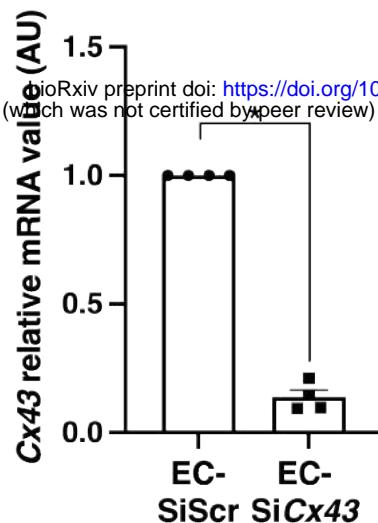
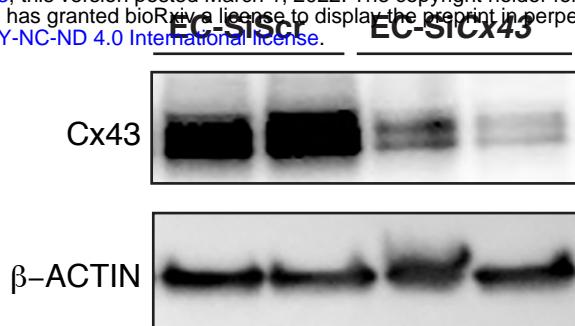
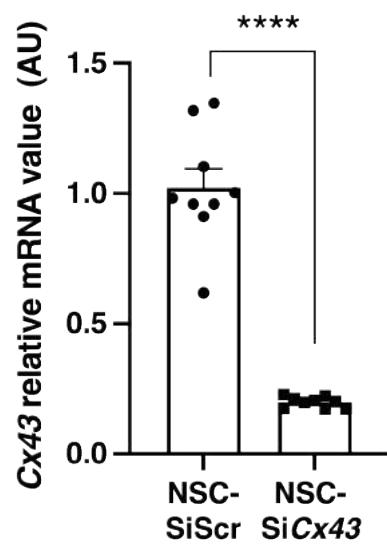
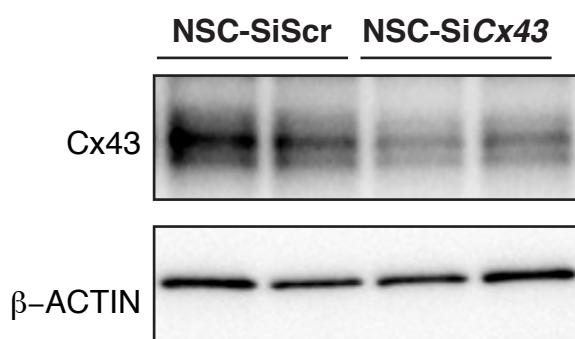
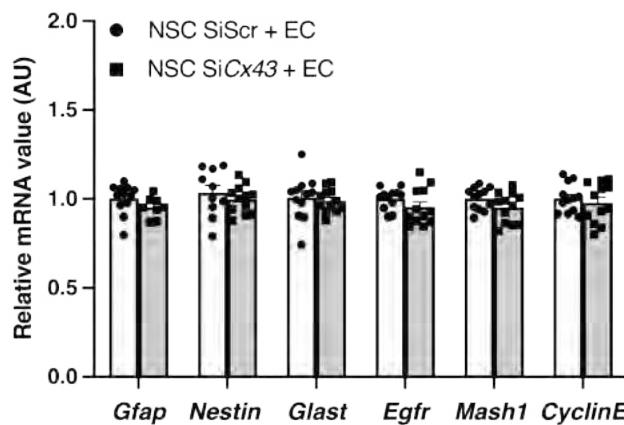
a**In vivo****b****In vitro**

Figure 7: Schematic representation of Cx43 regulation of NSCs.

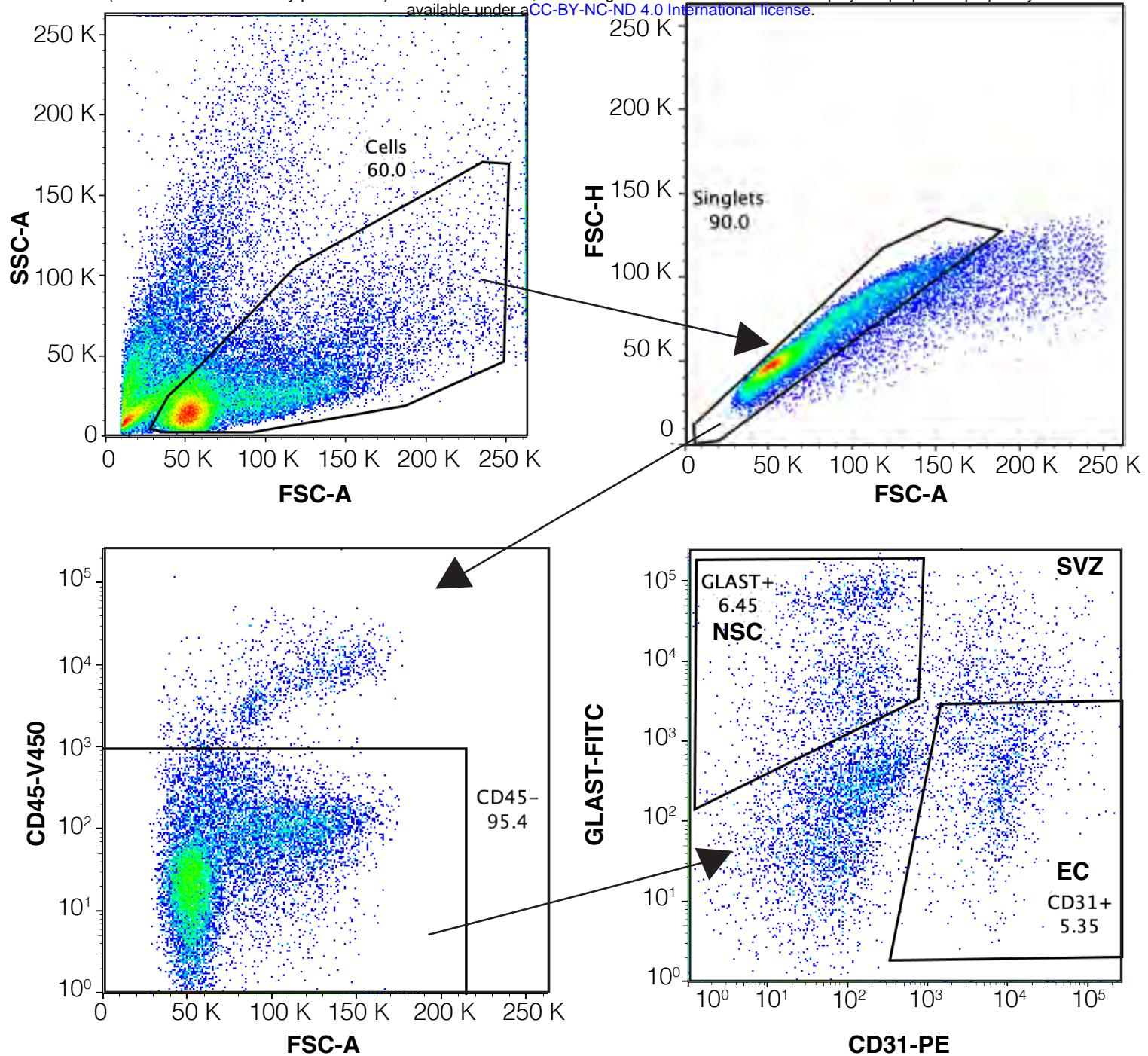
(a) *in vitro*: ECs and NSCs co-culture upregulates Cx43 expression. Cx43-mediated regulation of NSC quiescence and activation is dependent on Cx43 cytoplasmic tail and ERK activation. (b) *in vivo*: absence of Cx43-mediated interaction between ECs and NSCs increases the number of neuroblasts in the SVZ and RMS leading to an increase of newborn neurons in the OB. Absence of vascular Cx43 impairs SVZ neuroblasts re-population post depletion.

a**b****c****d****e**

Extended Figure 1: **(a)** qPCR analysis of *Cx43* mRNA level in EC treated with control siRNA (SiScr) or *Cx43* siRNA (siCx43) (n=4 different experiments). **(b)** Representative western blot of *Cx43* protein expression in EC treated with control siRNA (SiScr) or *Cx43* siRNA (siCx43). **(c)** qPCR analysis of *Cx43* mRNA level in NSC treated with control siRNA (SiScr) or *Cx43* siRNA (siCx43) (n=8 different experiments). **(d)** Representative western blot of *Cx43* protein expression in NSC treated with control siRNA (SiScr) or *Cx43* siRNA (siCx43). Data are mean \pm SEM. * p \leq 0.05, **** p \leq 0.0001. **(e)** qPCR analysis of quiescent and activated genes in NSC co-cultured with EC, where NSC are treated with control siRNA (siScr) or *Cx43* siRNA (SiCx43) (n=3 different experiments).

Extended Figure 2

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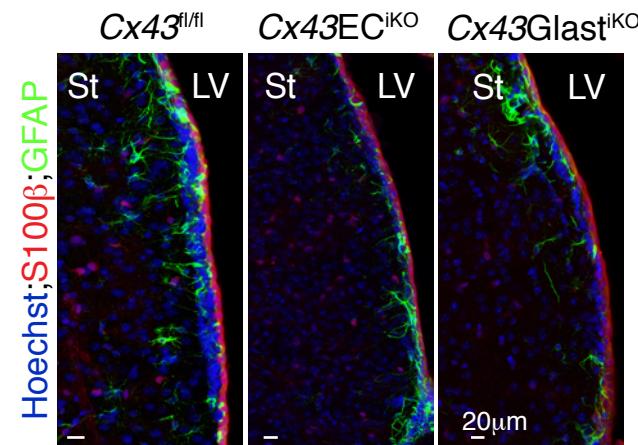
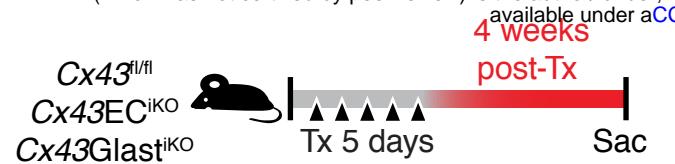


Extended Figure 2: Representative FACS plot showing the gating strategy for ECs and NSCs isolation from adult mouse brain SVZ. After excluding debris (top left panel) and doublets (top right panel), CD45⁻ cells are selected (bottom left panel) and CD31⁺Glast NSC are collected (bottom right panel). Percentages refer to the population of cells in the previous parent gate. Plots show 100,000 events. SSA: Side-scatter area, FSC: forward-scatter area, FSH: forward-scatter height.

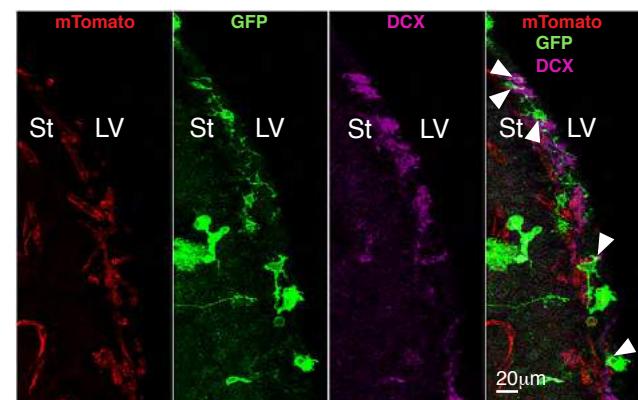
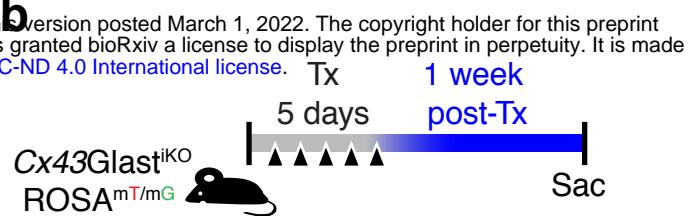
Extended Figure 3

a

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b

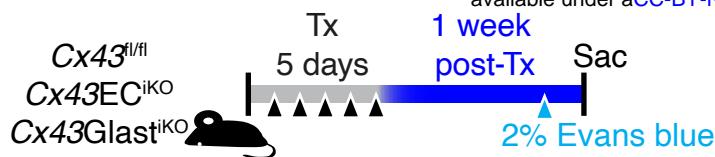


Extended Figure 3: (a) Timeline used for label retaining cell (LRC) protocol in *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice. **(b)** Representative confocal images of GFAP⁺SOX2⁺LRC in the SVZ of *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice. **(c)** Quantification of images shown in **(b)** (n=10 different sections from 2 animals). **(d)** Timeline used for long-term *Cx43* recombination in *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice. Bottom panel shows representative confocal images of S100 β ⁺GFAP⁺ cells in the SVZ of *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice. **(e)** Timeline used for short-term *Cx43* recombination in *Cx43Glast*^{iKO};Rosa^{mT/mG} mice. Bottom panel shows a representative confocal image of a coronal SVZ section showing presence of DCX⁺GFP⁺ neuroblasts (white arrowheads) in the population of GFP⁺ recombinant cells. St: Striatum; LV: Lateral ventricle. Data are mean \pm SEM. ** p \leq 0.01, **** p \leq 0.0001.

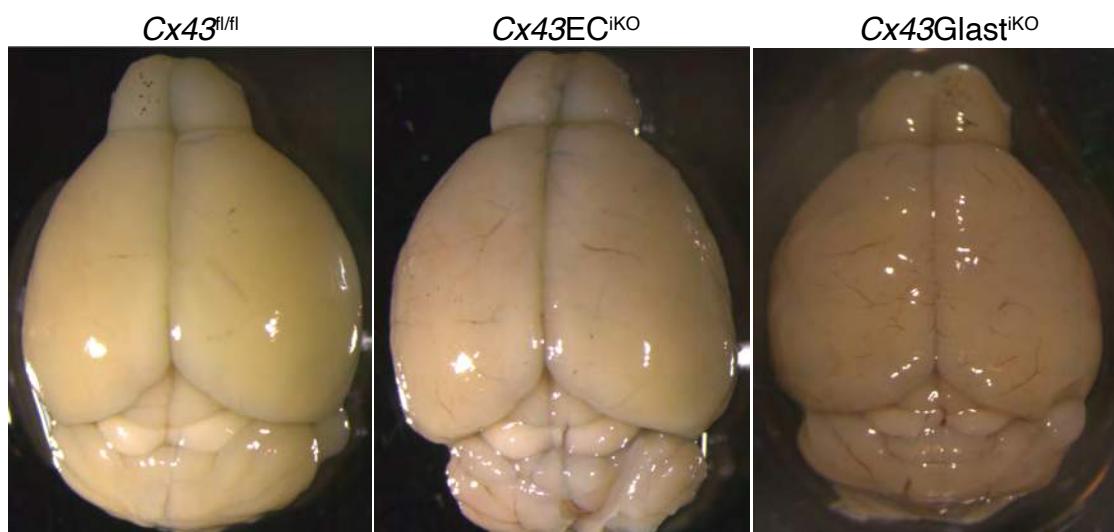
Extended Figure 4

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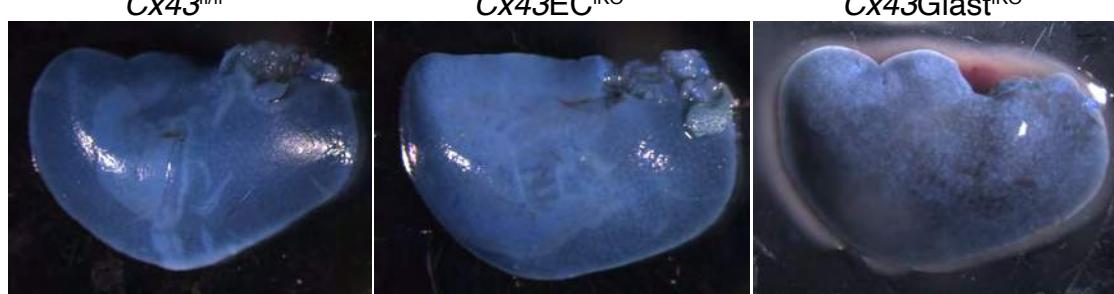
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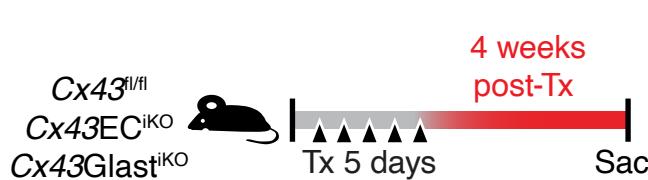
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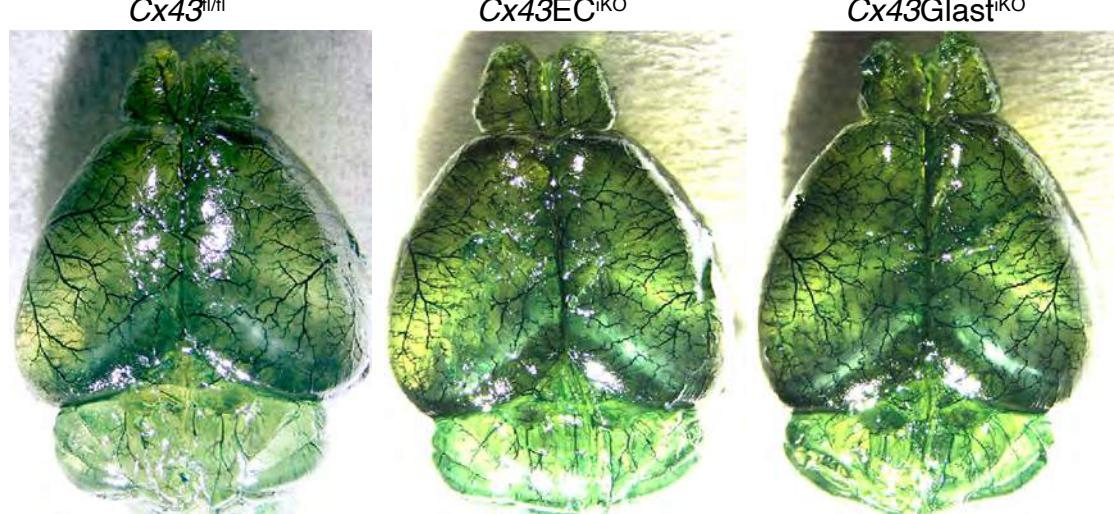
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d



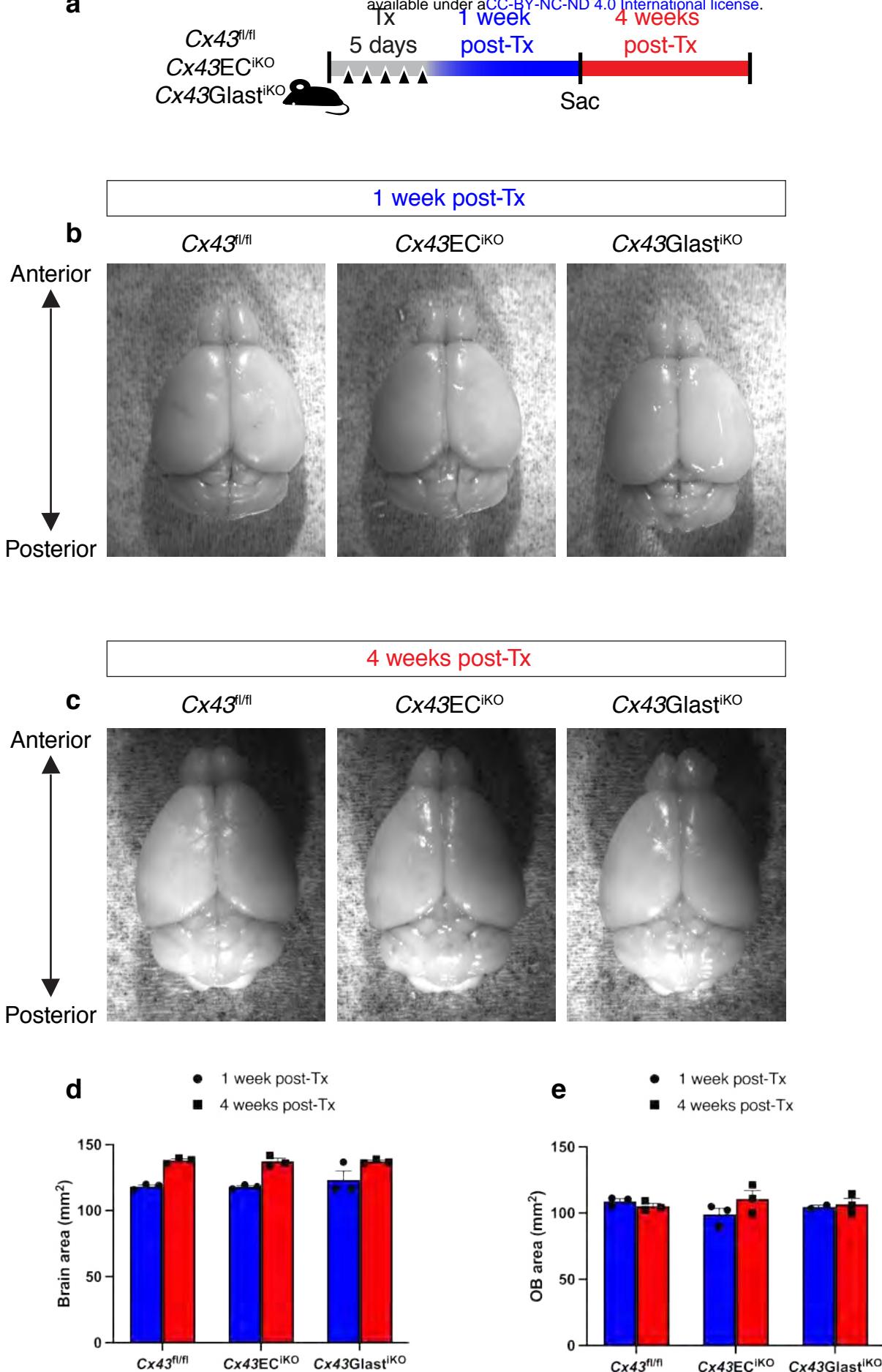
e



Extended Figure 4: (a) Timeline used to evaluate Evans blue permeability in *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice. (b) Representative brain images from *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice 24h post-2% Evans blue injection, showing absence of dye leakage. (c) Representative liver images from *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice 24h post-2% Evans blue injection showing dye uptake. (d) Timeline used to visualize brain microvasculature network organization in *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice. (e) Representative clarified brain images showing the microvasculature perfused with vascupaint green from *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO}.

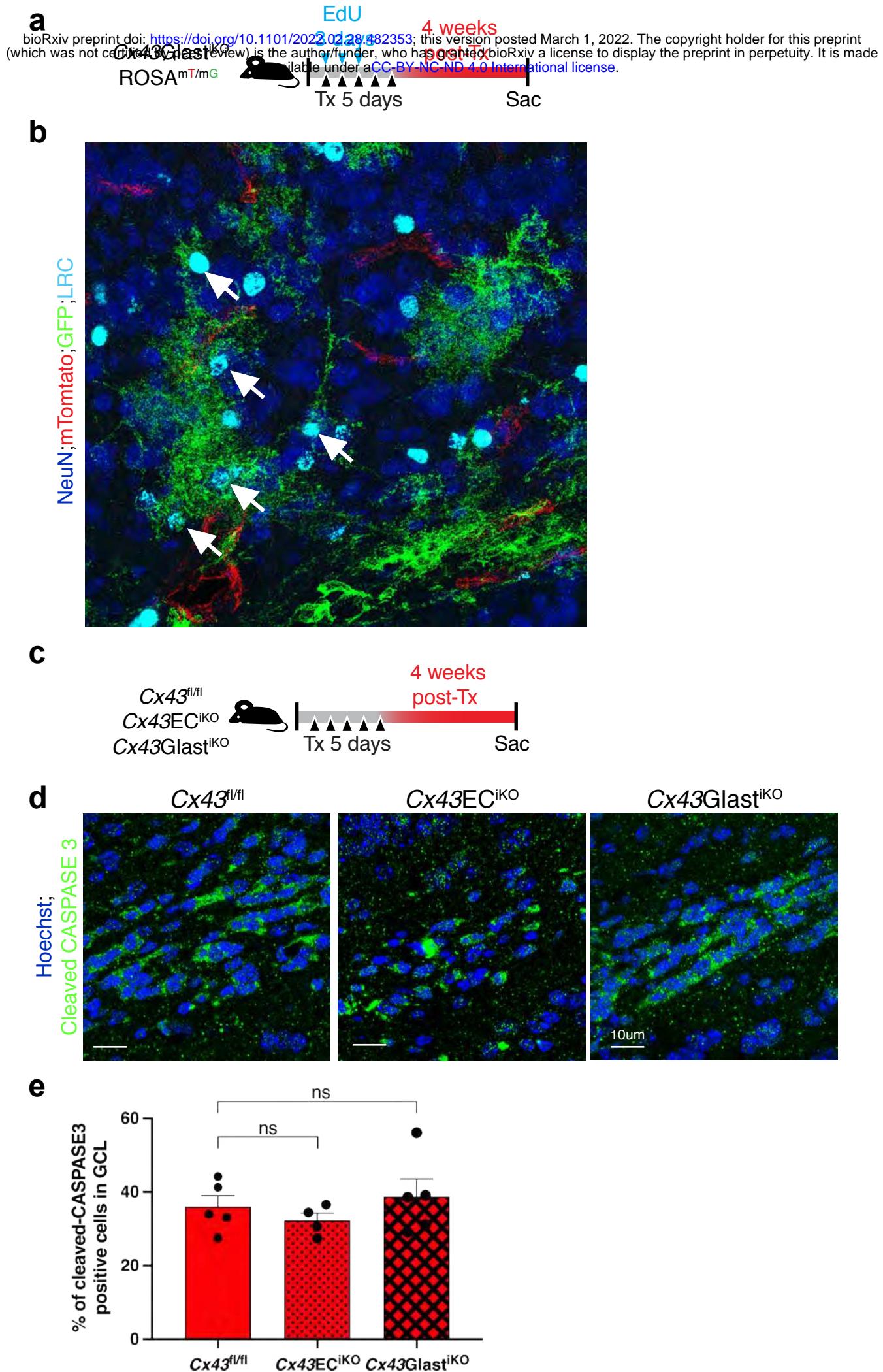
Extended Figure 5

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Extended Figure 5: (a) Timeline used to evaluate brain and OB areas of *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice. (b) and (c) Representative brain images from *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice 1-week and 4-weeks post-Tx injections respectively. (d) and (e) Bar graphs showing quantifications of brain and OB areas of *Cx43*^{fl/fl}, *Cx43EC*^{iKO} and *Cx43Glast*^{iKO} mice respectively at 1-week and 4-weeks post-Tx injections.

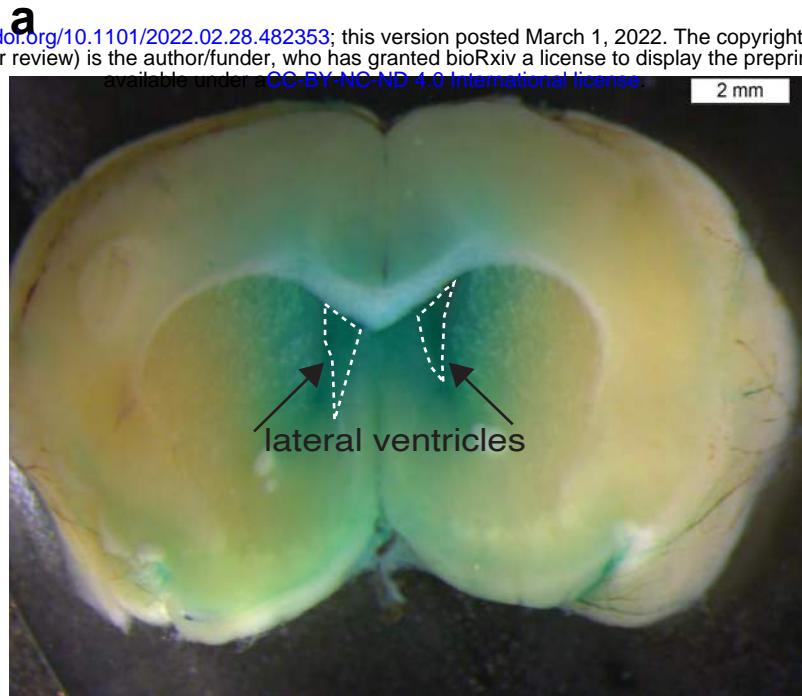
Extended Figure 6



Extended Figure 6: (a) Timeline used for *Cx43* recombination in *Cx43Glast^{iKO};Rosa^{mT/mG}* mice. (b) Representative confocal image of NeuN⁺LRC in the granule cell layer of the olfactory bulb of *Cx43Glast^{iKO};Rosa^{mT/mG}* mice. Note that NeuN⁺LRC are GFP⁺ (white arrows). (c) Timeline used for long-term *Cx43* recombination in *Cx43^{f/f}*, *Cx43EC^{iKO}* and *Cx43Glast^{iKO}* mice. (d) Representative images of cleaved CASPASE-3⁺ cells in the granule cell layer of the olfactory bulb of *Cx43^{f/f}*, *Cx43EC^{iKO}* and *Cx43Glast^{iKO}* mice. (e) Quantifications of images shown in (d) (n=4 different images analyzed from 1 animal per group). Data are mean \pm SEM. ns > ≤ 0.05 .

Extended Figure 7

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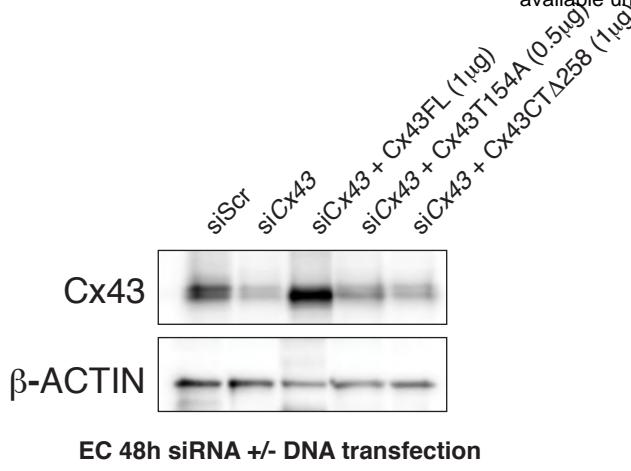


Extended Figure 7: Image of brain sagittal section of a mouse injected with fast green dye in the ipsi-lateral ventricle at the optimized coordinates (x:1.4, y:0.5, z:2.5) used to infuse AraC. Note that both lateral ventricles (enclosed in dashed lines) are green colored after dye infusion, demonstrating that the coordinates used target the SVZ.

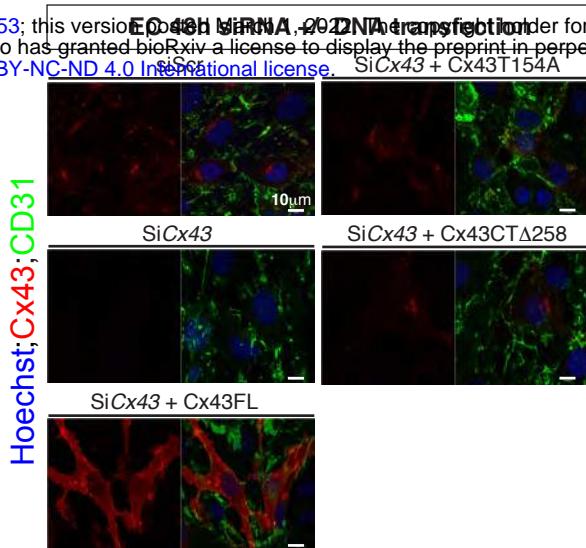
Extended Figure 8

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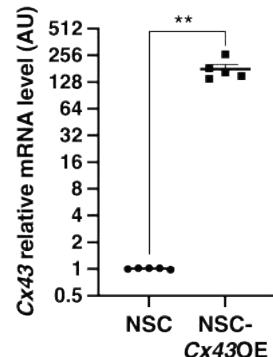
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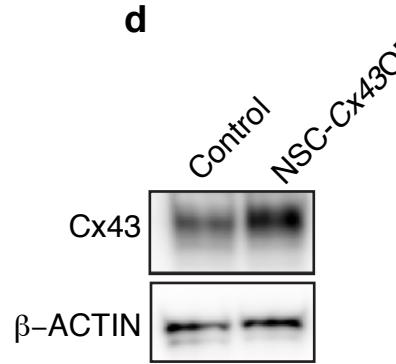
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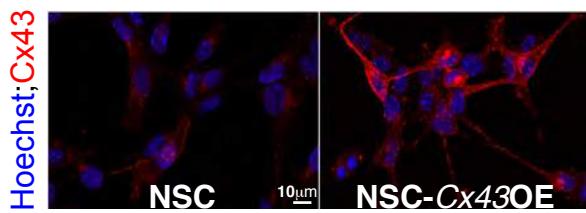
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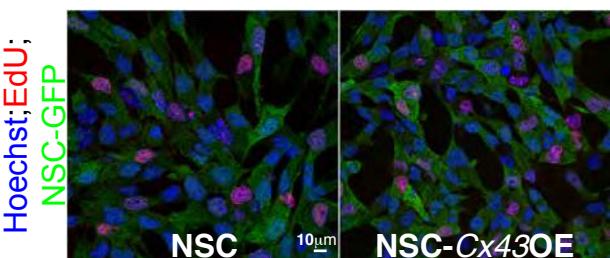
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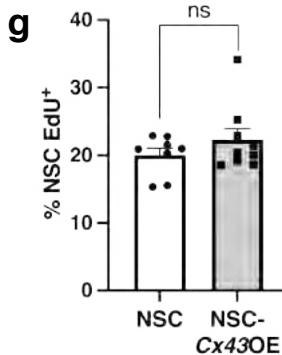
e



f



g



Extended Figure 8: (a) Representative western blot of Cx43 protein expression in ECs treated with control siRNA (SiScr), Cx43 siRNA (siCx43), SiCx43 followed by Cx43 full length construct (FL), SiCx43 followed by Cx43T154A or SiCx43 followed by Cx43CT Δ 258. (b) qPCR analysis of Cx43 mRNA level in NSC and NSC overexpressing Cx43 (NSC-Cx43OE) (n=5 different experiments). (c) Representative western blot of Cx43 protein expression in NSC and NSC-Cx43OE. (d) Representative confocal images of NSC and NSC-Cx43OE immuno-stained for Cx43. (e) Representative confocal images of NSC-GFP and NSC-GFP-Cx43OE 24h after EdU incorporation. (f) Quantification of images shown in (d) (n=4 different experiments). Data are mean \pm SEM. ns > 0.05, ** p < 0.01.