

1 **Nkx2.1 regulates the proliferation and cell fate of telencephalic astrocytes during**  
2 **embryonic development**

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22 **Abstract**

23 The homeodomain transcription factor Nkx2.1 controls cell differentiation of  
24 telencephalic GABAergic interneurons and oligodendrocytes. Here, we show that  
25 Nkx2.1 additionally regulates astrogliogenesis of the telencephalon from embryonic  
26 day (E) 14.5 to E16.5. Our work aims to identify the different mechanisms by which  
27 Nkx2.1 controls telencephalic astrogliogenesis. In *Nkx2.1*<sup>-/-</sup>, a drastic loss of  
28 astrocytes is observed which is not related to cell death. *In vivo* analysis using BrdU  
29 incorporation reveals that Nkx2.1 affects the proliferation of ventral neural stem cells  
30 that generate early astrocytes. *In vitro* neurosphere assays show that Nkx2.1  
31 additionally affects the differentiation step of Nkx2.1-derived astrocytes. Chromatin  
32 immunoprecipitation and *in vitro* co-transfection studies of a Nkx2.1-expressing  
33 plasmid indicate that Nkx2.1 binds to the promoter of astroglial differentiation gene  
34 GFAP, and regulates its expression. Hence, Nkx2.1 controls astroglial production  
35 spatiotemporally in embryos by regulating stem cell division and specification of the  
36 contributing Nkx2.1<sup>+</sup> precursors.

37

## 38 **Introduction**

39 Proper forebrain development is carried out by coordinated and regulated  
40 developmental events involving controlled cell proliferation, differentiation, and  
41 guided migration of neuronal and glial cells. Several spatiotemporally orchestrated  
42 molecular mechanisms underlie the successful patterning of the telencephalon  
43 (Guillemot et al., 2006, Long et al., 2009, Marin and Rubenstein, 2001, Puelles et al.,  
44 2000, Schuurmans et al., 2004, Schuurmans and Guillemot, 2002, Yun et al., 2001).  
45 Both dorsal and ventral telencephalons are demarcated by specific gene expression  
46 that regulates the generation of defined neuronal and glial populations. Dorsal  
47 progenitors express homeobox genes of the empty spiracles (*Emx1/Emx2*), and paired  
48 homeobox (*Pax6*) families, and *atonal*-related genes Neurogenin (*Ngn1/Ngn2*)  
49 whereas the ventral progenitors are known to exhibit expression of homeobox genes  
50 of the *Nkx* (*Nkx2.1*) and distal-less (*Dlx1/Dlx2*) families, *Gsh1/2*, and *achaete-scute*-  
51 related gene *Mash1* (Campbell, 2003, Guillemot, 2007b, Morrow et al., 2001, Qian et  
52 al., 2000, Rubenstein et al., 1998).

53 Broadly, amongst the neuronal population, the glutamatergic projection  
54 neurons have been shown to be primarily generated by dorsal telencephalic  
55 progenitors whereas the GABAergic interneurons originate from the ventral  
56 telencephalic progenitors (Kriegstein and Noctor, 2004, Marin et al., 2000, Marin and  
57 Rubenstein, 2001, Molyneaux et al., 2007). Amongst the glial population, the  
58 embryonic oligodendrocytes are produced in waves from the ventral telencephalic  
59 progenitors (Kessaris et al., 2006, Kessaris et al., 2008). On the other hand, the exact  
60 timing of generation and origin of the embryonic astroglial population is still a topic  
61 of active investigation. Embryonic astrocytes have been shown to be either generated  
62 from bipotential radial glia or from progenitor cells in the subventricular zone

63 (Levison and Goldman, 1993, Schmechel and Rakic, 1979, Guillemot, 2007a,  
64 Kriegstein and Alvarez-Buylla, 2009, Mori et al., 2005, Pinto and Gotz, 2007). In the  
65 dorsal telencephalon, astrocyte gliogenesis has been mostly documented to only occur  
66 after neurogenesis (after E17, in mice) when the bipotential radial glial cells of the  
67 dorsal pallium differentiate into astrocytes (Guillemot, 2007a, Mission et al., 1991,  
68 Rowitch and Kriegstein, 2010, Price and Thurlow, 1988, Cameron and Rakic, 1991,  
69 Lavdas et al., 1999, Schmechel and Rakic, 1979, Gotz and Huttner, 2005). Several  
70 indusium griseum (IG) glia, surrounding the CC, are also shown to originate from the  
71 radial glia of the dorsomedial pallium (Smith et al., 2006). The time of generation of  
72 some of the astrocytes occupying the CC midline region, however, is noted to be  
73 between E13 and postnatal day 2 (P2) with a peak at E14, much earlier than  
74 previously proposed (Shu et al., 2003). Furthermore, the postnatal astrocytes that  
75 occupy the cerebral cortex region are believed to originate from progenitor cells in the  
76 dorsolateral subventricular zone (SVZ) (Marshall et al., 2003). Recent evidence  
77 shows that a population of locally differentiated glia in the postnatal cortex instead  
78 constitute the primary source of astrocytes rather than the aforementioned SVZ  
79 progenitors (Ge et al., 2012). Since the glia play essential roles in guidance of  
80 forebrain commissures during embryonic brain development, hence, the detailed  
81 understanding of the point of origin(s) and exact timing of generation of the  
82 telencephalic astroglia is necessary.

83 Nkx2.1, a homeodomain transcription factor, was initially found to regulate  
84 the transcription of many thyroid-specific genes (Guazzi et al., 1990, Lazzaro et al.,  
85 1991, Sussel et al., 1999) and lung-specific genes (Boggaram, 2009, Hamdan et al.,  
86 1998). Furthermore, several cell cycle related genes such as *Notch1*, *E2f3*, *Cyclin B1*,  
87 *Cyclin B2* and *c-Met*, have been found to be bound by Nkx2.1 in developing

88 embryonic lungs (Tagne et al., 2012). In the brain, it is known to control during  
89 embryonic development, the specification of GABAergic interneurons and  
90 oligodendrocytes that populate the ventral and dorsal telencephalic region (Anderson  
91 et al., 2001, Corbin et al., 2001, Kessaris et al., 2006, Kimura et al., 1996, Marin and  
92 Rubenstein, 2001, Sussel et al., 1999). Loss of Nkx2.1 leads to ventral-to-dorsal  
93 respecification of the pallidum, and causes loss of GABAergic interneurons and  
94 oligodendrocytes in the dorsal telencephalic region (Kessaris et al., 2006, Kessaris et  
95 al., 2008, Sussel et al., 1999). Recently, we showed that during embryonic  
96 development Nkx2.1 also regulates the generation of astrocytes that populate the  
97 ventral telencephalon and participates to axonal guidance in the anterior commissure  
98 (Minocha et al., 2015a, Minocha et al., 2015b). We find that this Nkx2.1-derived  
99 astrocyte population are generated from three ventral telencephalic precursor regions,  
100 namely the medial ganglionic eminence (MGE), the anterior entopeduncular area  
101 (AEP)/ preoptic area (POA), and the triangular septal nucleus (TS) (Minocha et al.,  
102 2015a, Minocha et al., 2015b).

103 In this study, we found that Nkx2.1-derived astrocytes populate the corpus  
104 callosum (CC) and its surrounding regions in the embryonic dorsal telencephalon.  
105 The Nkx2.1-derived astrocytes are generated from E12.5 onwards with maximal  
106 production between E14.5-to-E16.5. Interestingly, by using *Nkx2.1*<sup>-/-</sup> mice, we  
107 observed that the functional ineffectiveness of the mutated Nkx2.1 (mut-Nkx2.1)  
108 leads to a drastic loss of astrocytes and polydendrocytes in the entire dorsal  
109 telencephalic region at the midline. Since the aforementioned Nkx2.1-derived cell  
110 loss is not accompanied with increased cell death, we further analyzed if cell  
111 proliferation of Nkx2.1<sup>+</sup> stem cells in the three ventral precursor regions (MGE,  
112 AEP/POA and TS) was affected. *In vivo* BrdU incorporation and *in vitro* neurosphere

113 differentiation assays showed that Nkx2.1 interestingly exerts its control over  
114 astroglial generation by controlling both the proliferation and differentiation capacity  
115 of the Nkx2.1<sup>+</sup> precursors. In addition, chromatin immunoprecipitation analysis  
116 indicated that the transcription factor Nkx2.1 binds to the promoter of astroglial  
117 differentiation gene, glial fibrillary acidic protein (*GFAP*). Co-transfection studies  
118 with *GFAP* promoter construct and tagged Nkx2.1 over-expression in HEK293 cells  
119 confirmed that Nkx2.1 could indeed regulate the expression of *GFAP* gene. Hence,  
120 Nkx2.1 regulates astroglial generation by regulating the proliferation and  
121 differentiation of the contributing Nkx2.1<sup>+</sup> precursors in ventral telencephalic zones.  
122 Thus, Nkx2.1 exhibits a multilevel control over the generation and differentiation of  
123 the telencephalic astroglia by spatially coordinating the astroglial generation from the  
124 three aforementioned precursor regions and temporally restricting maximal generation  
125 between E14.5-to-E16.5. Further analysis into the complete repertoire of genes  
126 regulated by Nkx2.1 can shed light about the glial and neuronal populations that play  
127 a defining role in shaping the brain.

128

129 **Results**

130

131 **Nkx2.1-derived astrocytes populate the dorsal telencephalon during development**

132 Nkx2.1-positive (Nkx2.1<sup>+</sup>) progenitors of the MGE, the AEP/POA and the septal  
133 nucleus contribute towards the production of embryonic GABAergic interneurons and  
134 oligodendrocytes that populate the ventral and dorsal telencephalon (Anderson et al.,  
135 2001, Corbin et al., 2001, Kessarar et al., 2006, Kimura et al., 1996, Marin and  
136 Rubenstein, 2001, Sussel et al., 1999). Our recent results have shown that Nkx2.1  
137 additionally regulates the production of astrocytes and polydendrocytes that populate  
138 the ventral telencephalon (Minocha et al., 2015a, Minocha et al., 2015b). Here,  
139 interestingly, further immunostaining against the subpallial transcription factor  
140 Nkx2.1 revealed strong expression in several differentiated cells within and  
141 surrounding the CC in the dorsal telencephalon from E16.5 to E18.5 too (n=8; Fig.  
142 1a-g). To further differentiate the Nkx2.1<sup>+</sup> cell types in the CC region, we made use  
143 of several cell-type specific transgenic strains and immunohistochemical analysis for  
144 neuronal and glial makers.

145       Firstly, to identify the GABAergic interneurons, we made use of the *Gad1-*  
146 *EGFP* knock-in mice, which express the green fluorescent protein (EGFP) in  
147 *GAD67<sup>+</sup>* GABAergic interneurons (Tamamaki et al., 2003) in combination with  
148 immunostaining against Nkx2.1. Coherent with previous observations showing down-  
149 regulation of Nkx2.1 expression in dorsal telencephalic GABAergic interneuron  
150 population (Nobrega-Pereira et al., 2008), at E16.5, none of the *Gad1-GFP<sup>+</sup>*  
151 interneurons of the CC and dorsal surrounding areas expressed Nkx2.1 (n=4; Fig. 1a-  
152 b; solid arrowheads in 1b).

153           Secondly, to ascertain if Nkx2.1<sup>+</sup> cells corresponded to polydendrocytes, we  
154 made use of the *Cspg4-Cre<sup>+</sup>/Rosa-EYFP* mice that express the yellow fluorescent  
155 protein (EYFP) in NG2<sup>+</sup> polydendrocytes (Nishiyama et al., 2009, Nishiyama et al.,  
156 2002, Minocha et al., 2015a). We found that the EYFP was expressed by Nkx2.1<sup>+</sup>  
157 progenitors of the MGE (n=3; Figure 1-figure supplement 1a-c, solid arrowheads in  
158 b-c). However, the EYFP<sup>+</sup> polydendrocytes stopped to express the Nkx2.1 protein  
159 outside ventral germinal zones and within the dorsal telencephalon (n=3; Figure 1-  
160 figure supplement 1g-i, open arrowheads in h-i). Hence, to further delineate the  
161 profile of Nkx2.1<sup>+</sup> cells in the CC and the surrounding areas, we performed  
162 immunostaining in wild-type embryos, against Nestin and GLutamate and ASpartate  
163 Transporter (GLAST) that are specific for post-mitotic astrocytes within the  
164 embryonic CC white matter (Shu et al., 2003). Interestingly, from E16.5 to E18.5,  
165 2/3<sup>rd</sup> of Nkx2.1<sup>+</sup> cells of the CC and surrounding regions were found to be GLAST<sup>+</sup>  
166 astrocytes (n=11; Fig. 1c-d, 1f-g, 2a-b, 2e, Figure 1-figure supplement 1h-i, solid  
167 arrowheads). After postnatal day 0 (P0), however, Nkx2.1 was strongly down-  
168 regulated and not detected anymore by immunohistochemistry in the astrocytes of the  
169 dorsal telencephalon (n=8, not shown). Nkx2.1 expression was never detected in any  
170 radial glial precursor cells of the glial wedge (GW) and of the dorsal telencephalic  
171 ventricular zone labeled for the aforementioned astrocytic markers (n=11; Figure 1-  
172 figure supplement 2).

173           Further analyses using tamoxifen-inducible *GLAST-Cre ERT<sup>TM</sup>/Rosa26-EYFP*  
174 mice, displayed the presence of many EYFP<sup>+</sup> early astrocytes outside the germinal  
175 zones and also within the CC and surrounding area from E16.5 to E18.5 (n=5; Fig.  
176 1e-j). Many of these early astrocytes visualized by the EYFP and GLAST co-staining

177 showed Nkx2.1 expression as well (n=5; CC in Fig. 1f-g and MGE in 1i-j, solid  
178 arrowheads).

179 Therefore, our previous and current extensive immunohistochemical analyses  
180 in combination with different transgenic strains reveal that the CC and the  
181 surrounding regions are populated with various Nkx2.1-derived glial cell types  
182 (summarized in Figure 1-figure supplement 3) (Minocha et al., 2015b). Presence or  
183 absence of Nkx2.1 protein expression primarily divides the Nkx2.1-derived glial  
184 classes into two major subtypes in both dorsal and ventral telencephalon — astrocyte-  
185 like or polydendrocyte-like. The Nkx2.1-derived astrocyte-like population is further  
186 sub-divided into two populations:  $GLAST^+/GFAP^+/Nkx2.1^+$  (orange) and  
187  $GLAST^+/GFAP^-/Nkx2.1^+$  (green) (Figure 1-figure supplement 3). The  
188 polydendrocyte-like population is further sub-divided into two populations:  
189  $S100\beta^+/NG2^+/Olig2^+/Nkx2.1^-$  (red) and  $S100\beta^-/NG2^+/Olig2^+/Nkx2.1^-$  (brown)  
190 populations (Figure 1-figure supplement 3). Interestingly, a subpopulation of  
191  $GLAST^+$  astrocyte-like population within the telencephalon (blue) are not Nkx2.1-  
192 derived and express  $Olig2^+$  cells (n=4; Fig. 2a-b and 2e; Figure 1-figure supplement  
193 3) (Minocha et al., 2015b).

194 Nkx2.1-derived astrocyte-like cells populate the CC region toward the end of  
195 embryonic period. Use of tamoxifen-inducible *GLAST-Cre ERT<sup>TM</sup>/Rosa26-EYFP*  
196 mice indicated the generation of several  $Nkx2.1^+/GLAST^+/EYFP^+$  astrocytes at a  
197 period beginning from E14.5 onwards as the tamoxifen injection was delivered at  
198 E14.5 (n=5; Fig. 1e-g). In addition, in order to further decipher the exact timing of  
199 generation of these  $Nkx2.1^+$  glial cells that occupy the CC region, we administered 5-  
200 bromo-2'-deoxyuridine (BrdU) injections to WT pregnant females bearing embryos at  
201 E12.5 (n=2), at E14.5 (n=2), and at E16.5 (n=2), and followed the extent of BrdU

202 incorporation at E18.5 by CC astrocytes co-expressing Nkx2.1 and astroglial markers,  
203 GLAST or GFAP (Fig. 3). Combined immunostaining revealed the presence of few  
204 BrdU<sup>+</sup>/Nkx2.1<sup>+</sup>/GLAST<sup>+</sup> and BrdU<sup>+</sup>/GFAP<sup>+</sup> embryonic astrocytes in the CC when  
205 BrdU injection was delivered at E12.5 (Fig. 3a-c and j, solid arrowheads). The bulk of  
206 the BrdU<sup>+</sup>/Nkx2.1<sup>+</sup>/GLAST<sup>+</sup> and BrdU<sup>+</sup>/GFAP<sup>+</sup> embryonic astrocytes was observed  
207 when BrdU injection was delivered at E14.5 (Fig. 3d-f and k, solid arrowheads), and  
208 at E16.5 (Fig. 3h-i and l, solid arrowheads). Therefore, the majority of Nkx2.1-  
209 derived astrocytes occupying the CC region are produced between E14.5 to E16.5.

210 Hence, these results indicate that Nkx2.1 expression is only maintained in the  
211 astrocyte population of the CC region from E14.5 to E16.5.

212

### 213 **Nkx2.1 controls gliogenesis in embryonic telencephalon**

214 To further investigate the function of Nkx2.1 in regulating embryonic gliogenesis, we  
215 performed immunohistochemistry for astroglial (GLAST and GFAP) and  
216 polydendroglial (NG2) markers in control and *Nkx2.1*<sup>-/-</sup> embryos expressing  
217 inactivated truncated Nkx2.1 (mut-Nkx2.1) at E18.5 (Fig. 4). For control, we made  
218 use of both homozygous (*Nkx2.1*<sup>+/+</sup>) and heterozygous (*Nkx2.1*<sup>+/-</sup>) mice. In control  
219 mice, GLAST<sup>+</sup> (n=2) or GFAP<sup>+</sup> (n=4) astroglia, and NG2<sup>+</sup> (n=5) polydendrocytes  
220 were clearly visible in the CC and its surrounding regions, in the medial cortical area  
221 as well as in the septum (Fig. 4a-c and 4e). In contrast, we observed a drastic loss of  
222 astroglia (n= 2 stained for GLAST; n=3 stained for GFAP) and polydendrocytes (n=4)  
223 in all midline dorsal regions in *Nkx2.1*<sup>-/-</sup> mice (Fig. 4f-h and 4j). However, Nkx2.1  
224 inactivation did not affect the number and organization of radial glia within the GW,  
225 in accordance with the absence of Nkx2.1 expression in these glial cell types  
226 (compare Fig. 4a,b and 4f,g). Quantitative measurements made with the astrocyte

227 marker, GFAP (Fig. 4k) and polydendrocyte marker, NG2 (Fig. 4l) confirmed the  
228 drastic loss (70 to 100%) of astroglia and polydendrocytes in the midline dorsal  
229 telencephalic areas in *Nkx2.1*<sup>-/-</sup> mice. There was a drastic disappearance of GFAP<sup>+</sup>  
230 astrocytes in the CC and its surrounding areas (IG and MZG) in the *Nkx2.1*<sup>-/-</sup> embryos  
231 (n=3) compared to control embryos (n=4) (p-value=0.0056 for CC, 0.0216 for IG,  
232 0.0067 for MZG, Fig. 4k). Interestingly, analysis with GFAP also revealed the loss of  
233 a subpopulation of GFAP<sup>+</sup> radial glial precursors within the ventral telencephalon, in  
234 the mutant POA\* and MGE\* VZ (Fig. 4i, p-value=0.0496 for MGE and 0.0247 for  
235 POA; Fig. 4k). Additionally, there was also a near complete loss (99 to 100%) of  
236 NG2<sup>+</sup> polydendrocytes in the medial cortical areas of *Nkx2.1*<sup>-/-</sup> embryos (n=3)  
237 compared to control embryos (n=5) (p-value=0.0334 for CC medial and 0.0191 for  
238 CC lateral; Fig. 4j and 4l).

239 Furthermore, since we identified different *Nkx2.1*-derived glial cell  
240 populations, both astrocyte-like and polydendrocyte-like, this prompted us to  
241 investigate in further the function of Nkx2.1 in glia specification while considering  
242 these different glial cell types (Figure 2). To this purpose, we performed  
243 immunostaining for Olig2 and GLAST on telencephalic CC sections from both  
244 controls (n=4) and *Nkx2.1*<sup>-/-</sup> (n=2) mice. In the *Nkx2.1*<sup>-/-</sup> CC, a significant reduction  
245 (around 70%) in the cell density of GLAST<sup>+</sup>/Olig2<sup>-</sup> astrocytes was observed when  
246 compared to the WT (p-value= 0.0297), while no differences were detected for  
247 GLAST<sup>+</sup>/Olig2<sup>+</sup> astroglial cell densities (p-value= 0.4469) (Fig. 2a-e). The  
248 GLAST/Olig2<sup>+</sup>/ polydendrocyte population was nearly completely lost (around 98%)  
249 in the CC (p-value= 0.0242; Fig. 4e). Hence, these results show that Nkx2.1 regulates  
250 both the GLAST<sup>+</sup>/Olig2<sup>-</sup> astroglial and GLAST<sup>-</sup>/Olig2<sup>+</sup> polydendroglial populations

251 but not the GLAST<sup>+</sup>/Olig2<sup>+</sup> astroglial population (also illustrated in Figure 1-figure  
252 supplement 3).

253 In order to exclude the possibility of cell death being the central reason for the  
254 marked decrease in the number of glial cells we observed, we analyzed the controls  
255 (n=4 for CC region; n= 5 for POA region) and *Nkx2.1*<sup>-/-</sup> (n=6 for CC region; n=10 for  
256 POA region) brains at E16.5 for cleaved-caspase 3, a key biomarker for apoptosis  
257 (Figure 4-figure supplement 1a-d and 1i). We also performed terminal  
258 deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay  
259 which detects DNA fragmentation that results from different cell death processes  
260 (n=16 for CC in controls, n=22 for CC in knockouts; n=6 for POA in controls, n=5  
261 for POA in knockouts; n=10 for MGE in controls, n= 11 for MGE in knockouts; n=7  
262 for SEP in controls, n=14 for SEP in knockouts; Figure 4-figure supplement 1e-h and  
263 1j). The DNA binding dye, Hoechst was also used to visualize the pyknotic nuclei and  
264 in addition, to determine if there are any differences in nuclear size or nuclear  
265 morphology between the WT and mutant brains. The quantification of the absolute  
266 number of dying cells, labeled by the cleaved-caspase 3 (Figure 4-figure supplement  
267 1i), and by TUNEL staining (Figure 4-figure supplement 1j), revealed no significant  
268 differences between the *Nkx2.1*<sup>-/-</sup> brains and the control brains in any of the observed  
269 telencephalic regions namely the CC, the MGE, the POA or the septum (p-value=  
270 0.1225 for CC and 0.4618 for POA with cleaved caspase 3 staining; p-value= 0.7934  
271 for CC, 0.8193 for POA, 0.4032 for MGE, and 0.4879 for SEP with TUNEL). The  
272 size and morphology of the cell nuclei was comparable in both WT and mutant brains,  
273 with no significant differences observed.

274           These results show that the glial cells occupying the CC are under the  
275 regulation of Nkx2.1. Also, the observed loss of astrocytes and polydendrocytes in the  
276 *Nkx2.1*<sup>-/-</sup> telencephalon is not due to the glial cell death.

277

278 **Nkx2.1 regulates the proliferation of astrocyte ventral progenitors in embryonic**  
279 **brains**

280 The loss of specified glia in *Nkx2.1*<sup>-/-</sup> mice may be owing to insufficient proliferation  
281 of ventral glial Nkx2.1-precursors of the progenitor zones, namely MGE, AEP/POA  
282 and TS. To investigate further, we performed double immunohistochemical staining  
283 for Nkx2.1 and for the radial glia/astrocytic marker, GLAST on coronal sections of  
284 the precursor regions in both *Nkx2.1*<sup>+/+</sup> or *Nkx2.1*<sup>+/-</sup> control (n=4) and *Nkx2.1*<sup>-/-</sup> (n=4)  
285 mice brains at E16.5. Likely both, the mutated Nkx2.1 (mut-Nkx2.1) and the WT  
286 Nkx2.1 proteins are similarly recognized by the anti-Nkx2.1 antibody indicating that  
287 the mutated protein conserves an intact epitope sufficient for recognition by the  
288 antibody (Corbin et al., 2003).

289           In the VZ, the SVZ and the mantle zone of the control MGE, many GLAST<sup>+</sup>  
290 precursors and differentiated astroglia co-expressed Nkx2.1 (Fig. 5a-c and 5h, solid  
291 arrowheads) whereas in the germinal and mantle zones of the mutant MGE\* of  
292 *Nkx2.1*<sup>-/-</sup> mice, only few GLAST<sup>+</sup> precursors and astroglia co-labeled for the mut-  
293 Nkx2.1 were observed (Fig. 5d-f, solid arrowheads). This observed difference may be  
294 attributed to the fact, as shown before, that although a MGE-like structure forms in  
295 the mutant (called as MGE\*), it has been re-specified to a more dorsal LGE-like fate  
296 (Sussel et al., 1999). Similar reduction of cells labeled for GLAST and the mut-  
297 Nkx2.1 were seen in the mutant POA\* of *Nkx2.1*<sup>-/-</sup> mice (Fig. 5i-j). The quantitative

298 analyses revealed a drastic and significant decrease of the total cells (50 to 85%) and  
299 GLAST<sup>+</sup> precursors (45 to 86%) expressing the mutated Nkx2.1 in the VZ, SVZ of  
300 MGE\*, POA\* and TS\* (p-value < 0.0001 in the VZ of MGE, POA and TS in Fig. 5k-  
301 l and p-value=0.0139 for the SVZ of MGE in Fig. 5k). Consequently, the number of  
302 GLAST<sup>+</sup> differentiated astrocytes co-expressing the mut-Nkx2.1 in the parenchyma  
303 (striatum, LPOA/LH, septum) of the *Nkx2.1*<sup>-/-</sup> (n=4) was severely decreased (60 to  
304 80%) as compared to control mice (n=4) (p-value<0.0001 for striatum, LPOA and  
305 septum in Fig. 5l). Thus, these results indicate that the mutation of Nkx2.1 results in  
306 the severe loss of precursors in the mutant mice brains.

307 To ascertain the cell proliferation status of precursors at the germinal zones,  
308 we made use of the S-phase marker, BrdU. The rate of cell proliferation was studied  
309 in *Nkx2.1*<sup>+/+</sup> (n=8) or *Nkx2.1*<sup>+/-</sup> (n=3) control and *Nkx2.1*<sup>-/-</sup> (n=8) mice with a principal  
310 attention at E16.5 when the bulk of embryonic telencephalic glia is generated. In the  
311 control brains, both in the VZ and SVZ of the MGE, numerous Nkx2.1<sup>+</sup> precursors  
312 were co-labeled with BrdU (Fig. 6a-c, solid arrowheads). For quantification, we used  
313 n=4 control and n=4 knockouts (Fig. 6g-j). In the mutant MGE\* of *Nkx2.1*<sup>-/-</sup> mice, we  
314 observed a significant reduction in the BrdU<sup>+</sup> progenitors of the VZ and SVZ  
315 compared to the control MGE (compare Fig. 6c to 6f; p-value < 0.0006 in the VZ of  
316 MG and p-value <0.0064 in the SVZ of MGE in Fig. 6g). Intriguingly, an increase in  
317 number of total BrdU<sup>+</sup> cells was seen in the POA\* VZ and SVZ regions of the  
318 *Nkx2.1*<sup>-/-</sup>. Nonetheless, remaining BrdU<sup>+</sup> precursors only very rarely expressed the  
319 mutated Nkx2.1 in both the mutant MGE\* and POA\* regions (Fig. 6h). The number  
320 of dividing cells labeled for the mut-Nkx2.1 was severely reduced (75 to 80%) in the  
321 *Nkx2.1*<sup>-/-</sup> brains (p-value <0.0001 in the VZ of MGE, POA and in the SVZ of MGE,  
322 POA in Fig. 6h). The mutation of Nkx2.1 results in the incapacity of Nkx2.1-derived

323 precursors to divide (p-value <0.0001 in the VZ and SVZ of MGE and p-value  
324 <0.0005 in the SVZ of POA in Fig. 6i). By contrast, dividing cells that did not express  
325 the mut-Nkx2.1 in the *Nkx2.1*<sup>-/-</sup> brains were either not affected or up-regulated in the  
326 mutant MGE\* and POA\* regions (Fig. 6j).

327 Altogether, these observations indicate that the transcription factor Nkx2.1  
328 controls the proliferation step of the original Nkx2.1<sup>+</sup> precursors in the MGE and the  
329 POA, the subpallial domains that majorly generate early embryonic astroglia.

330

### 331 **Nkx2.1 regulates the differentiation of Nkx2.1-derived astroglia in embryonic** 332 **brains**

333 Next, we aimed to analyze the specification of Nkx2.1<sup>+</sup> precursors capable of  
334 generating early astroglial cells by observing the MGE- and the POA-derived  
335 neurosphere differentiation at E14.5. After 7 days *in vitro* (DIV), control MGE and  
336 POA neurospheres were able to differentiate and generate two cell types of the brain  
337 (Arsenijevic et al., 2001) which were GFAP<sup>+</sup> mature astrocytes (Fig. 7a-b) and  $\beta$ III-  
338 tubulin<sup>+</sup> post-mitotic neurons (not shown). GFAP<sup>+</sup> astrocytes were observed to be  
339 uniformly dispersed on the entire surface of the spheres (Fig. 7a-b). Using  
340 immunohistochemistry for Nkx2.1, we found that in the neurospheres derived from  
341 control MGE and POA, Nkx2.1 was expressed in the nucleus of about 50% of the  
342 GFAP<sup>+</sup> astroglia (Fig. 7b and e; solid arrowheads). Thereafter, *in vitro* differentiation  
343 of E14.5 *Nkx2.1*<sup>-/-</sup> mutant MGE\* and POA\*-derived neurospheres revealed that,  
344 precursors expressing the mut-Nkx2.1 stopped to differentiate into mut-  
345 Nkx2.1<sup>+</sup>/GFAP<sup>+</sup> astroglia (Fig. 7c-d). Quantification showed that the mutation of  
346 Nkx2.1 in the MGE\* and POA\* neurospheres of *Nkx2.1*<sup>-/-</sup> induced a significant

347 decrease of mut-Nkx2.1<sup>+</sup>/GFAP<sup>+</sup> astroglia (p-value <0.0001 in the MGE and POA  
348 neurospheres in Fig. 7e). While more than 50% of differentiated GFAP<sup>+</sup> astroglia  
349 expressed Nkx2.1 in control neurospheres, less than 10% of GFAP<sup>+</sup> astroglia  
350 expressed mut-Nkx2.1 in mutant neurospheres (Fig. 7e). It indicates that in mutant  
351 MGE\* and POA\* neurospheres, mut-Nkx2.1<sup>+</sup> precursors have nearly completely lost  
352 the capacity to differentiate into astroglia.

### 353 **Nkx2.1 directly regulates the expression of the GFAP astroglial regulatory gene**

354 It is clear from our above mentioned results that the astroglial cell populations of the  
355 embryonic telencephalon are derived from Nkx2.1<sup>+</sup> progenitors, and Nkx2.1 regulates  
356 the astrocyte precursor cells proliferation and differentiation. In order to ascertain if  
357 the transcription factor Nkx2.1 regulates expression by binding onto the promoter  
358 sequences of the astroglial GFAP regulatory gene in the brain, we performed  
359 chromatin immunoprecipitation assay on lysates of E16.5 embryonic brains. Firstly,  
360 we searched for DNA elements matching the consensus NK2 family binding sequence  
361 [GNNCACT(T/C)AAGT(A/G)(G/C)TT] (Guazzi et al., 1990) in the upstream  
362 promoter regions of the regulatory gene GFAP and of the negative control gene Ngn2  
363 that regulates dorsal precursors (Fode et al., 2000). In the absence of the complete  
364 consensus binding sequence, the core binding sequence T(C/T)AAG was chosen for  
365 analysis. As a positive control, we included Lhx6 in our analysis for which the site for  
366 the binding of Nkx2.1 is already known (Du et al., 2008). Secondly, after shortlisting  
367 the position of putative Nkx2.1 binding sites, primers for the sequence flanking all the  
368 shortlisted binding sites (up to three) were made. We then performed the PCR on the  
369 crosslinked and sonicated DNA pulled down using an anti-Nkx2.1 monoclonal  
370 antibody. The amplification of a putative Nkx2.1 binding sequence located in the  
371 PCR product within the astroglial gene for GFAP (Fig. 8a) was found to be positive in

372 the brain samples chromatin immunoprecipitated with the Nkx2.1 antibody, however,  
373 as expected no positive interaction was detected for Ngn2 (Fig. 8c). Also, the  
374 amplification of the PCR product comprising the already known Nkx2.1 binding site  
375 within the Lhx6 promoter was positive upon immunoprecipitation with the anti-  
376 Nkx2.1 antibody (Fig. 8b). Furthermore, the PCR fragment(s) amplified for the GFAP  
377 promoter contained the core sequence CTCAAGT of the Nkx2.1 consensus binding  
378 sequence. Thus, these results suggest that, *in vivo*, Nkx2.1 binds the promoter region  
379 of the GFAP astroglial regulatory gene, which contains the aforementioned highly  
380 conserved core-binding sequence of the consensus binding site.

381         Thereafter, to confirm the direct influence of the binding of Nkx2.1 to this  
382 upstream binding sequence towards the transcription of GFAP astroglial regulatory  
383 gene, we performed co-transfection studies in the HEK293 cells. We made use of the  
384 expression plasmid *pDRIVE-mGFAP*, which contains the LacZ reporter under the  
385 control of the mouse upstream -1679 bp GFAP promoter sequence (and includes the  
386 putative Nkx2.1 binding site identified above), and the *pCAG-Nkx2.1-IRES-Tomato*  
387 plasmid constitutively over-expressing the Nkx2.1 protein and the Tomato protein  
388 under the control of the *pCAG* promoter. Co-transfection of both plasmids in the  
389 HEK293 cells resulted in robust expression of the LacZ reporter (Fig. 8g-i).  
390 Contrastingly, almost no expression was apparent upon transfection of the *pDRIVE-*  
391 *mGFAP* plasmid with the control *pCAG-IRES-Tomato* plasmid lacking the *Nkx2.1*  
392 cDNA (Fig. 8d-f). Thus, these results suggest that the activation of the *GFAP*  
393 promoter fragment requires the presence of Nkx2.1, which most probably recognizes  
394 the binding sequence identified by us.

395            These results, altogether, show that the *Nkx2.1* homeobox gene indeed  
396 regulates proliferation and differentiation of the astroglia that occupy the CC region  
397 during late embryonic ages.

398

## 399 **Discussion**

400 Nkx2.1 has been implicated during the specification of GABAergic interneurons and  
401 oligodendrocytes that occupy the embryonic telencephalon (Anderson et al., 2001,  
402 Corbin et al., 2001, Kessarar et al., 2006, Kimura et al., 1996, Marin and Rubenstein,  
403 2001, Sussel et al., 1999). Recently, our group has shown that Nkx2.1 controls the  
404 production of GABAergic interneurons, astrocytes and polydendrocytes that populate  
405 the embryonic ventral telencephalon (Minocha et al., 2015a, Minocha et al., 2015b).  
406 In this study, our results unravel that Nkx2.1 also regulates the generation of dorsal  
407 astroglia that populate the corpus callosum and its surrounding regions during late  
408 embryonic stages. Nkx2.1 mediates its control over astroglia through regulation of  
409 both proliferation and differentiation of Nkx2.1<sup>+</sup> precursors present in the ventral  
410 progenitor regions, namely the MGE, the AEP/POA and the TS (Minocha et al.,  
411 2015a, Minocha et al., 2015b). By controlling the production of neuron and glia that  
412 populate the entire telencephalon, Nkx2.1 is a key factor for brain shaping during  
413 embryonic development.

414

### 415 **Multilevel regulation of embryonic astroglialogenesis by Nkx2.1**

416 The Nkx2.1-derived cell population in embryos is broadly divided into astrocyte-like  
417 and polydendrocyte-like based on their expression profile, and is generated maximally  
418 between E14.5-to-E16.5. Only Nkx2.1<sup>+</sup> astrocyte-like cells that are GLAST<sup>+</sup> and/or  
419 GFAP<sup>+</sup> continue to maintain Nkx2.1 expression while other Nkx2.1-derived cells that  
420 are NG2<sup>+</sup>/Olig2<sup>+</sup> polydendrocyte-like no longer express Nkx2.1 as soon they  
421 differentiate. Loss of Nkx2.1 function in *Nkx2.1*<sup>-/-</sup> mice leads to a drastic reduction in  
422 the number of both astroglia and polydendrocytes in the midline dorsal (CC, IG,  
423 MZG) and within the ventral telencephalic (mutant MGE\* and POA\*, and septum)

424 regions. The loss of astroglia and polydendrocytes is not accompanied with a  
425 concomitant increase in apoptotic cells. Hence, the results indicated that the loss  
426 might be due to incapacity of the precursors to generate astroglia and  
427 polydendrocytes. Indeed, further analyses revealed that the loss of glia is  
428 accompanied with a decrease of *Nkx2.1*-derived precursor division capacity and  
429 astroglial differentiation. Accordingly, we observed a drastic decrease in presence of  
430 total cells and GLAST<sup>+</sup> precursors expressing the mut-Nkx2.1 in the VZ, SVZ of  
431 mutant MGE\*, mutant POA\* and the TS region, and of the GLAST<sup>+</sup> differentiated  
432 astrocytes expressing the mut-Nkx2.1 in the parenchyma (striatum, LPOA/LH,  
433 septum) of the *Nkx2.1*<sup>-/-</sup> compared to the WT mice. The decreased presence of  
434 precursors and differentiated astroglial population was accompanied with reduced  
435 proliferative status of the BrdU<sup>+</sup> dividing cells labeled for mut-Nkx2.1 in VZ and  
436 SVZ of mutant MGE\*, mutant POA\* and the septal nucleus region of *Nkx2.1*<sup>-/-</sup> mice  
437 compared to the WT precursors. Additionally, *in vitro* differentiation of E14.5 *Nkx2.1*<sup>-/-</sup>  
438 mutant MGE\*, POA\*-derived neurospheres revealed that, after Nkx2.1 inactivation,  
439 the progenitors were unable to differentiate into GFAP<sup>+</sup> astroglia expressing the mut-  
440 Nkx2.1<sup>+</sup> significantly though they still retained the capacity to generate post-mitotic  
441 neurons. Hence, the reduction in number of astroglia and polydendrocytes can be  
442 attributed to a deficit in proper proliferation and differentiation of precursors in three  
443 subpallial domains in the absence of Nkx2.1. Chromatin immunoprecipitation analysis  
444 also suggests that the Nkx2.1 might mediate this control by direct activation of  
445 astroglial gene promoter region, GFAP here.

446 Thus, Nkx2.1 regulates the generation and specification of dorsal  
447 telencephalic astroglia through a multilevel control that involves (i) control over

448 proliferation of Nkx2.1<sup>+</sup> precursors, (ii) regulation of differentiation of Nkx2.1<sup>+</sup>  
449 precursors, and lastly, (iii) transcriptional control over astroglial gene like GFAP.

450

451 **Nkx2.1 is important for several aspects of proper brain development during**  
452 **embryogenesis**

453 Previous reports from our and other groups have shown that not only is Nkx2.1  
454 important for regional specification of the ventral telencephalic regions, MGE and  
455 POA, it is also essential for generation of a wide spectrum of Nkx2.1-derived lineages  
456 including GABAergic interneurons, polydendrocytes and astrocytes that populate  
457 both the dorsal and ventral telencephalon beginning from E12.5 (Anderson, 2001,  
458 Corbin et al., 2001, Du et al., 2008, Kessarar et al., 2006, Kessarar et al., 2008,  
459 Kimura et al., 1996, Marin et al., 2000, Marin and Rubenstein, 2001, Marin et al.,  
460 2010, Minocha et al., 2015a, Minocha et al., 2015b, Nobrega-Pereira et al., 2008,  
461 Sussel et al., 1999, Xu et al., 2008). Maximal generation of Nkx2.1-derived cell types  
462 occurs around E14.5-to-E16.5, a period characterized by several key developmental  
463 events, including midline fusion, and bordering the formation of corpus callosum and  
464 anterior commissure and also blood vessel network (Adams and Alitalo, 2007,  
465 Larrivee et al., 2009, Minocha et al., 2015a, Minocha et al., 2015b, Paul et al., 2007,  
466 Richards et al., 2004). Loss of Nkx2.1 leads to ventral-to-dorsal transformation of the  
467 pallidum (Sussel et al., 1999), together with drastic reduction in Nkx2.1-derived cell  
468 population leading to structural abnormalities in anterior commissure and blood  
469 vessel network (Minocha et al., 2015a, Minocha et al., 2015b). Also, the reduced  
470 GABAergic neuronal localization in the Nkx2.1<sup>-/-</sup> mice lead to callosal axon  
471 branching and outgrowth defects in the CC tract (Niquille et al., 2013).

472           This study shows that Nkx2.1 is able to perform its vast range of roles through  
473 regulation of both proliferation and differentiation of Nkx2.1<sup>+</sup> precursors. It appears  
474 that Nkx2.1 mediates some (or all) of these effects through transcriptional regulation  
475 of target genes, such as astroglial gene GFAP characterized in this study.

476           Previous reports have shown that Nkx2.1 regulates the transcription of many  
477 genes of the thyroid (Guazzi et al., 1990, Lazzaro et al., 1991, Sussel et al., 1999) and  
478 activates pulmonary-surfactant (Boggaram, 2009), as well as pituitary gland genes  
479 (Hamdan et al., 1998). Moreover, it has been shown *in vitro*, that Nestin might be a  
480 target of *Nkx2.1* (Lonigro et al., 2001). Another group has also seen transcriptional  
481 patterns of regulation by Nkx2.1 in early (E11.5) and late (E19.5) mouse lung  
482 development (Tagne et al., 2012). Interestingly, in mouse lungs, Nkx2.1 also directly  
483 regulates the cell cycle effectors and its loss alters cell cycle progression (Tagne et al.,  
484 2012). In the ventral telencephalon, loss of Nkx2.1 function also affects the  
485 proliferation of precursors expressing the mut-Nkx2.1. Hence, it is probable that  
486 Nkx2.1 displays some functional conservation in brain, thyroid, pituitary, and lung —  
487 the four Nkx2.1<sup>+</sup> identified tissues.

488           Complex cellular and molecular interactions between glia, neurons and  
489 guidance cues produced by them govern the formation of the midline structures such  
490 as corpus callosum and anterior commissure. Several Nkx2.1-derived glial and  
491 neuronal populations populate these aforementioned structures, and further  
492 understanding of the mode of regulation mediated by Nkx2.1 can help better  
493 understand the formation of dorsal and ventral telencephalic regions.

494

495

496

## Methods

### 497 **Animals**

498 All studies on mice of either sex have been performed in compliance with the national  
499 and international guidelines. For staging of embryos, midday of the day of vaginal  
500 plug formation was considered as embryonic day 0.5 (E0.5). Wild-type mice  
501 maintained in a CD-1/SWISS genetic background were used for developmental  
502 analysis of the CC. We used wild-type (+/+) and homozygous mutant *Nkx2.1* mice  
503 (Flames et al., 2007, Kimura et al., 1996, Sussel et al., 1999), which are referred as  
504 *Nkx2.1*<sup>+/+</sup> and *Nkx2.1*<sup>-/-</sup> in this work. We used heterozygous *GAD67-GFP* knock-in  
505 mice, described in this work as *Gad1-EGFP* knock-in mice (Tamamaki et al., 2003).  
506 *Gad1-EGFP* knock-in embryos could be recognized by their GFP fluorescence. PCR  
507 genotyping of these lines was performed as described previously (Niquille et al.,  
508 2009). We used *GLAST-Cre ERT*<sup>TM</sup> (purchased from Jackson Laboratory: Tg(Slc1a3-  
509 cre/ERT)1Nat/J) transgenic mice. We used *Nkx2.1-cre* (Xu et al., 2008) and *Cspg4-*  
510 *cre* (Jackson Laboratory: B6;FVB-Tg(*Cspg4-cre*)1Akik/J) (Zhu et al., 2008)  
511 transgenic mice that have been described previously. The reporter mouse *Rosa26R-*  
512 *Enhanced yellow fluorescent protein (EYFP)* (Srinivas et al., 2001) was used to  
513 reliably express EYFP under the control of the Rosa26 promoter upon Cre-mediated  
514 recombination.

515 For the induction of CreERT, Tamoxifen (20 mg/ml, Sigma, St Louis, MO)  
516 was dissolved at 37°C in 5 ml corn oil (Sigma, St Louis, MO) pre-heated at 42°C for  
517 30 minutes. A single dose of 4 mg (250-300 µl) was administered to pregnant females  
518 by oral gavaging.

519

## 520 **Immunocytochemistry**

521 Embryos were collected after caesarean section and quickly killed by decapitation.  
522 Their brains were dissected out and fixed by immersion overnight in a solution of 4%  
523 paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. Brains were  
524 cryoprotected in a solution of 30% sucrose in 0.1 M phosphate buffer (pH 7.4), frozen  
525 and cut in 50 µm-thick coronal sections for immunostaining.

526 Mouse monoclonal antibodies were: BrdU (Monosan, Am. Uden,  
527 Netherlands) and GFAP (Chemicon). Rabbit polyclonal antibodies were: cleaved-  
528 caspase 3 (Chemicon), GFAP (DAKO, Carpinteria, CA); GFP (Molecular Probes,  
529 Eugene, OR); NG2 (Chemicon); Nkx2.1 (Biopat, Caserta, Italy); Olig2 (Millipore);  
530 Anti-β galactosidase (or LacZ) (Rockland); and RFP (Labforce MBL). Guinea pig  
531 antibody was: GLAST (Chemicon, Temecula, CA). Chicken antibody was: GFP  
532 (Aves). Goat antibody was: Anti-β galactosidase (or LacZ) (Biogenesis).

533 a) Fluorescence immunostaining was performed as follows: non-specific binding was  
534 blocked with 2% normal horse serum in PBS 1X solution with 0.3% Triton X-100 for  
535 preincubation and incubations. The primary antibodies were detected with Cy3-  
536 conjugated (Jackson ImmunoResearch laboratories, West Grove, PA) and Alexa488-,  
537 Alexa594- or Alexa647-conjugated antibodies (Molecular Probes, Eugene, OR).  
538 Sections were counterstained with Hoechst 33258 (Molecular Probes), mounted on  
539 glass slides and covered in Mowiol 4-88 (Calbiochem, Bad Soden, Germany).

540 b) DAB immunostaining was performed as follows: Endogenous peroxidase reaction  
541 was quenched with 0.5% hydrogen peroxide in methanol, and non-specific binding  
542 was blocked by adding 2% normal horse serum in Tris-buffered solutions containing  
543 0.3% Triton X-100 for preincubation and incubations. The primary antibodies were

544 detected with biotinylated secondary antibodies (Jackson ImmunoResearch, West  
545 Grove, PA) and the Vector-Elite ABC kit (Vector Laboratories, Burlingame, CA).  
546 The slices were mounted on glass slides, dried, dehydrated, and covered with Eukitt.

547

#### 548 **BrdU tracing studies**

549 To label cells in the S-phase of the cell cycle at the suitable embryonic stages (E12.5,  
550 E14.5 and E16.5), the pregnant female mice were injected intraperitoneally with a  
551 solution of 8 mg/ml of 5-bromo-2'-deoxyuridine (BrdU; Sigma, St Louis, MO) in PBS  
552 (0.15 M NaCl, 0.1 M phosphate buffer, pH = 7.4) to a final concentration of 50 mg/kg  
553 body weight. To trace the division rate of the subpallial precursors, the pregnant  
554 females were sacrificed 1-2 hours post-injection. To trace the date of genesis of the  
555 CC astrocytes, the pregnant females were sacrificed when embryos were E18.5. The  
556 BrdU was revealed by DAB or fluorescence immunostaining (as mentioned above)  
557 after a treatment with 2 M HCl for 30 min at room temperature.

558

#### 559 **Imaging**

560 DAB stained sections were imaged with a Zeiss Axioplan2 microscope equipped with  
561 10×, 20× or 40× Plan neofluar objectives and coupled to a CCD camera (Axiocam  
562 MRc 1388x1040 pixels). Fluorescent-immunostained sections were imaged using  
563 confocal microscopes (Zeiss LSM 510 Meta, Leica SP5 or Zeiss LSM 710 Quasar)  
564 equipped with 10×, 20×, 40×oil Plan neofluar and 63×oil, 100×oil Plan apochromat  
565 objectives. Fluorophore excitation and scanning were done with an Argon laser 458,  
566 488, 514 nm (blue excitation for GFP and Alexa488), with a HeNe laser 543 nm

567 (green excitation for Alexa 594 and CY3), with a HeNe laser 633 nm (excitation for  
568 Alexa 647 and CY5) and a Diode laser 405 nm (for Hoechst-stained sections). Z-  
569 stacks of 10-15 planes were acquired for each CC coronal section in a multitrack  
570 mode avoiding crosstalk.

571 All 3D Z stack reconstructions and image processing were performed with  
572 Imaris 7.2.1 software. To create real 3D data sets we used the mode “Surpass”. The  
573 colocalization between two fluorochromes was calculated and visualized by creating a  
574 yellow channel. Figures were processed in Adobe Photoshop© CS4 and CS5 and  
575 schematic illustrations in Supplementary Figure 2 were produced using Adobe  
576 Illustrator© CS4.

577

## 578 **Quantifications**

### 579 a) *Glial cell population analysis*

580 In 50 µm thick brain sections of *Nkx2.1<sup>+/+</sup>* and *Nkx2.1<sup>-/-</sup>* embryos at E18.5, the  
581 astroglial cells were labeled for GFAP and polydendroglial cells were labeled for  
582 NG2. Cells were counted in the CC, IG, MGE, MZG and POA regions from at least 4  
583 brains per condition. The cell densities were reported per surface unit area (number of  
584 cells/mm<sup>2</sup>). The quantification was done using NeuroLucida 9.0 and NeuroLucida 9.0  
585 Explorer© software.

586 In 50 µm thick brain sections of *Nkx2.1<sup>+/+</sup>* and *Nkx2.1<sup>-/-</sup>* embryos at E18.5, the  
587 astroglial cells that were labeled for Olig2 or both Olig2 and GLAST were counted in  
588 the CC mid from at least 2 brains per condition. Olig2 staining labeled the glial cell  
589 bodies while GLAST labeled both the cell bodies and processes. The cell densities

590 were determined in the medial and lateral part of the CC. The cell densities were  
591 reported per volume unit (number of cells/mm<sup>3</sup>). The quantification was done using  
592 Imaris® 7.2.1 software.

593

594 b) *Nkx2.1*<sup>+</sup> and *GLAST*<sup>+</sup> or *BrdU*<sup>+</sup> cell number analyses

595 Pregnant female mice were injected intraperitoneally with a solution of 8 mg/ml of 5-  
596 bromo-2'-deoxyuridine in PBS to a final concentration of 50 mg/kg body weight. To  
597 trace the division rate of the subpallial precursors, the pregnant females were  
598 sacrificed 2 hours post-injection. Embryos were collected after caesarean section and  
599 quickly killed by decapitation. Their brains were dissected out and fixed by  
600 immersion overnight in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer  
601 (pH 7.4) at 4°C. In 50 µm thick brain sections of *Nkx2.1*<sup>+/+</sup> and *Nkx2.1*<sup>-/-</sup> embryos at  
602 E16.5, *Nkx2.1*<sup>+</sup> cells, *BrdU*<sup>+</sup> dividing cells and *GLAST*<sup>+</sup> precursors or post-mitotic  
603 astroglial cells of the MGE, POA and TS were counted in the VZ, SVZ and in the  
604 parenchyma of each region, from at least 4 brains per condition. *Nkx2.1* and *BrdU*  
605 staining labeled the cell bodies while *GLAST* labeled both the cell bodies and  
606 processes. The percentage of *Nkx2.1*-derived dividing precursors or post-mitotic glial  
607 cells, were determined as follows: In each sub region, and for each condition, a  
608 sample of at least four different Z-stacks was acquired at 100x magnification by using  
609 a Leica SP5 confocal microscope. The Z-stacks comprised of 10 planes that were  
610 acquired in a multitrack mode avoiding any crosstalk. Thereafter, in order to exclude  
611 the possibility of quantifying the same cells more than once, snapshots of only 3  
612 planes (from the acquired 10 planes), were taken with Imaris® 7.2.1 software  
613 (Bitplane Inc.) and analyzed.

614 The quantification of Nkx2.1, BrdU, GLAST and Hoechst staining was done on each  
615 snapshot separately by using Neurolucida© 9.0 and Neurolucida 9.0 Explorer©  
616 software.

617

618 c) *Neurospheres differentiation analysis*

619 MGE- and POA-derived neurospheres were obtained from Nkx2.1<sup>+/+</sup> and Nkx2.1<sup>-/-</sup>  
620 E14.5 embryos. After 7 DIV, the neurospheres were differentiated and  
621 immunostained as mentioned above. Two different brains were used for each  
622 condition and were labeled for Nkx2.1, GFAP and  $\beta$ III tubulin. Cell nuclei were  
623 counterstained with Hoechst. For each condition, a total of at least 5 different Z-stacks  
624 in 5 different neurospheres were acquired at 100x magnification by using a Leica SP5  
625 microscope. The percentage of Nkx2.1<sup>+</sup>/GFAP<sup>+</sup> differentiated astrocytes and  
626 Nkx2.1<sup>+</sup>/ $\beta$ III tubulin<sup>+</sup> differentiated neurons were counted directly on the Z-stacks by  
627 using Imaris® 7.2.1 software.

628

629 d) *Cell death analysis*

630 In brain sections of Nkx2.1<sup>+/+</sup> and Nkx2.1<sup>-/-</sup> embryos at E16.5, apoptotic cells labeled  
631 for either cleaved-caspase 3 or for TUNEL were counted in the CC, MGE, SEP, and  
632 POA from at least 2 brains per condition. 50  $\mu$ m thick brain sections were used for  
633 cleaved-caspase 3 staining whereas 10  $\mu$ m thick brain sections were utilized for  
634 TUNEL staining. Cell nuclei were counterstained with Hoechst. For each condition,  
635 at least 5 different Z-stacks were obtained at 100x magnification by using a Leica SP5  
636 microscope. The number of apoptotic nuclei were counted and reported as an absolute

637 number per section (the surface area of one section was 24119.332  $\mu\text{m}^2$ ). The  
638 quantification was done using NeuroLucida 9.0 and NeuroLucida 9.0 Explorer©  
639 software.

#### 640 **Neurosphere generation and microscopical analysis**

641 The protocol has been adapted from Arsenijevic *et al.*, 2001.

##### 642 a) Primary culture and sphere passaging

643 The brains of embryos at developmental stage E14.5 were collected as described  
644 above. They were carefully removed from the skull into ice-cold sterile dissecting  
645 medium (MEM 1X) complemented with Glucose 1M (5ml/100ml). Thereafter, the  
646 brains were embedded in low melting point Agarose 3% (LMP-Agar, Gibco) at 37°C,  
647 and cut into 250  $\mu\text{m}$  thick slices using a vibratome (Leica© VT 1000 S). The sections  
648 were collected in the ice-cold dissecting medium. The areas of interest (MGE, POA  
649 and SEP) were dissected out using two tungsten needles under a stereomicroscope  
650 (Leica© MZ16F). The dissected pieces of tissue were then collected into 1ml ice-cold  
651 sterile Hormone Mix Medium (MHM 1X) supplemented with Penicillin (50 U/ml)  
652 and Streptomycin (50 U/ml) (GIBCO). The Hormone Mix Medium is a growing  
653 medium containing DMEM and F-12 nutrient (1:1), glucose (0.6%), glutamine (2  
654 mM), sodium bicarbonate (3 mM), HEPES buffer (5 mM), transferrin (100 mg/ml),  
655 insulin (25  $\mu\text{g/ml}$ ), progesterone (20 nM), putrescine (60  $\mu\text{M}$ ), selenium chloride (30  
656 nM) (Avery *et al.*). Brain tissue pieces were mechanically dissociated under sterile  
657 conditions with a fire-polished pipette in the Hormone Mix Medium. The pipette was  
658 rinsed before the dissociation of each new region.

659 The dissociated cells were then grown in Hormone Mix Medium  
660 complemented with Pen/Strep and EGF in 6-well dishes (Nunc Surface, NUNC

661 Brand Products, Nalge Nunc International) at a concentration of around  $10^4$ - $10^5$  cells  
662 per 1 ml and 4 ml per dish. After 6-7 days *in vitro* (DIV) at a temperature of 37°C in a  
663 5% CO<sub>2</sub> atmosphere, the sphere cultures were expanded. Primary spheres were  
664 dissociated mechanically and cells were plated at the density of  $2 \times 10^6$  cells for 40 ml  
665 in a flask (Nunclon Surface, NUNC Brand Products, Nalge Nunc International).  
666 Sphere passages were done every 7 DIV, by spheres dissociation and transfer of  
667  $2 \times 10^6$  cells to a new 40 ml flask.

668

#### 669 b) Differentiation of spheres

670 After 7 DIV, the neurospheres of optimum size were chosen under a stereomicroscope  
671 (Nikon©) to be transferred individually and plated onto poly-L-ornithine coated  
672 coverslips in 24-well plates (Nunclon Surface, NUNC Brand Products, Nalge Nunc  
673 International). Each coverslip contained about ten spheres and 1 ml of Hormone Mix  
674 Medium supplemented with Pen/Strep and 2% fetal bovine serum (FBS).

675

#### 676 c) Immunofluorescence on differentiated Neurospheres

677 After 7 DIV, the neurospheres were fixed in 4% PFA for 20 minutes and  
678 permeabilized with 0.3% triton/PBS1X for 3 minutes. Coverslips were incubated with  
679 primary antibodies diluted in PBS containing 10% NHS for 2 hours at room  
680 temperature, followed by secondary fluorescent antibodies for 45 minutes at 37° and  
681 Hoechst staining for 5 minutes.

682

## 683 **Chromatin Immunoprecipitation**

684 Chromatin immunoprecipitation was conducted on E16.5 brain samples according to  
685 the instructions provided by the manufacturer (Upstate, 17-295), using 2 $\mu$ g of a  
686 mouse anti-Nkx2.1 monoclonal antibody (MS699-P, Lab Vision). For crosslinking,  
687 1% PFA was used. For sonication, six bursts of 45 seconds ON (30% power) and 30  
688 second OFF were given, and samples were kept on ice during the whole sonication  
689 process. Mouse Genome Assembly data mm9 was used to map sites.

690 A 391 bp PCR fragment of the *Lhx6* promoter that includes a Nkx2.1 binding  
691 sequence at position –240 bp relative to the putative transcriptional start site was  
692 identified using primers 5'-ttgtaccgagagtaggagaagg and 5'-gtcctaactttgtagtggcattt.

693 A 206 bp PCR fragment of the *GFAP* promoter that includes a putative  
694 Nkx2.1 binding sequence (ctcaagt) at position –838 bp relative to the putative  
695 transcriptional start site was found to be a positive binding target and was identified  
696 using primers 5'- tggataagaggccacagagg and 5'- cctctcccctgaatctctcc.

697 Primers against two fragments of the *Neurogenin2* promoter region,  
698 comprising of the core Nkx2.1 binding consensus sequence (tcaag), were made. 1)  
699 Primers 5'-cgggattctgactctcactaattc and 5'-aatggttctaaagctctgttgg were designed to  
700 amplify a 410 bp PCR fragment with the core consensus Nkx2.1 binding sequence at  
701 position –668 bp relative to the putative transcriptional start site. 2) Primers 5'-  
702 cgggattctgactctcactaattc and 5'-aatggttctaaagctctgttgg were designed to amplify  
703 another 352 bp PCR fragment with the core consensus Nkx2.1 binding sequence at  
704 position –4073 bp relative to the putative transcriptional start site.

705

## 706 **Transfection of HEK293 cells**

707 A suspension of HEK-293 cells adapted to serum-free growth medium was  
708 plated at  $1 \times 10^6$  cells in 4 ml media in a 60mm plate. For formation of the  
709 transfection complexes, 3:1 ratio of FuGENE® HD Transfection Reagent ( $\mu$ l) :  
710 plasmid DNA ( $\mu$ g) was prepared and used for transfection. The study was performed  
711 by co-transfecting an expression plasmid for constitutive over-expression of Nkx2.1  
712 (*pCAG-Nkx2.1-IRES-Tomato*) or a control plasmid (*pCAG-IRES-Tomato*) with the  
713 *pDRIVE-mGFAP* plasmid containing the GFAP promoter region in front of the LacZ  
714 reporter gene. Transfection complexes were formed by mixing 2  $\mu$ g of each of the two  
715 plasmids with 12  $\mu$ l of Fugene transfection reagent and 188  $\mu$ l of Optimem reduced  
716 serum media. The mix was incubated at room temperature for 20 minutes and  
717 thereafter, added to the cell plates. The cell plates were kept in the 37°C incubator and  
718 gene expression analysis was done after 24-48 hours of transfection. Fluorescence  
719 immunostaining was done to visualize the presence and level of *LacZ* expression.  
720 Tomato signal was visible by direct fluorescence, however, for a clearer visualization  
721 of Tomato signal, an anti-RFP immunostaining was done. The method to do  
722 fluorescence immunostaining has been described above.

723

## 724 **Statistical analysis**

725 The results from all quantifications were analyzed with the aid of Statview software  
726 (SAS Institute Inc.). For all analysis, values from at least three independent  
727 experiments were first tested for normality and the variance of independent  
728 populations were tested for equality. Values that followed a normal distribution were

729 compared using Student's *t*-test. Values that did not follow a normal distribution were

730 compared using Mann-Whitney non-parametric test.

731

732 **Atlas and nomenclature**

733 The neuroanatomical nomenclature is based on the “Atlas of the prenatal mouse

734 brain” (Schambra et al., 1991).

735

736 **Abbreviations list**

737	AEP	Anterior entopeduncular area
738	BrdU	5-bromo-2'-deoxyuridine
739	CC	Corpus callosum
740	CCi	Cingulate cortex
741	CI	Cingulate bundle
742	Cre-ERT <sup>tm</sup>	Tamoxifen inducible Cre recombinase fused to the ligand
743		binding domain of the estrogen receptor
744	Cspg4	Chondroitin sulfate proteoglycan 4 (also known as NG2)
745	DIV	Day in vitro
746	E	Embryonic day
747	EGFP	enhanced green fluorescent protein
748	EYFP	enhanced yellow fluorescent protein
749	GABAergic	$\gamma$ -aminobutyric acidergic
750	Gad1	Glutamate decarboxylase 1 (also known as GAD67)
751	<i>Gad1-EGFP</i>	Gad1-EGFP knock-in mouse
752	GFAP	Glial fibrillary acidic protein
753	GLAST	Glutamate-aspartate transporter
754	GP	Globus pallidus
755	HIC	Hippocampal commissure
756	IG	Indusium griseum
757	IRES	Internal ribosome entry site
758	IZ	Intermediate zone
759	KO	knockout
760	LGE	Lateral ganglionic eminence

761	LH	Lateral hypothalamus
762	Lhx6	LIM homeodomain (LIM-hd) gene
763	LPOA	lateral preoptic area
764	LV	Lateral ventricle
765	MGE	Medial ganglionic eminence
766	MZ	Marginal zone
767	MZG	Midline zipper glia
768	NG2	Neuron-glial antigen 2
769	Ngn2	Neurogenin2
770	<i>Nkx2.1</i>	NK2 homeobox 1
771	<i>Nkx2.1-Cre</i>	Mouse with the Cre recombinase under control of the <i>Nkx2.1</i>
772		promoter
773	Olig2	Oligodendrocyte transcription factor
774	pCAG	promoter constructed from following sequences:
775		(C) cytomegalovirus early enhancer element,
776		(A) promoter, the first exon and intron of chicken beta-actin
777		gene,
778		(G) the splice acceptor of the rabbit beta-globin gene
779	POA	Preoptic area
780	RMS	Rostral migratory stream
781	<i>Rosa-EYFP</i>	Rosa26-lox-STOP-lox-EYFP reporter mouse
782	S100 $\beta$	Small EF-hand calcium and zinc binding protein
783	SEP	Septum
784	ST	Striatum
785	SVZ	Subventricular zone

786	TS	Triangular septal nucleus
787	TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
788	VZ	Ventricular zone
789	WT	wild-type
790		
791		

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800

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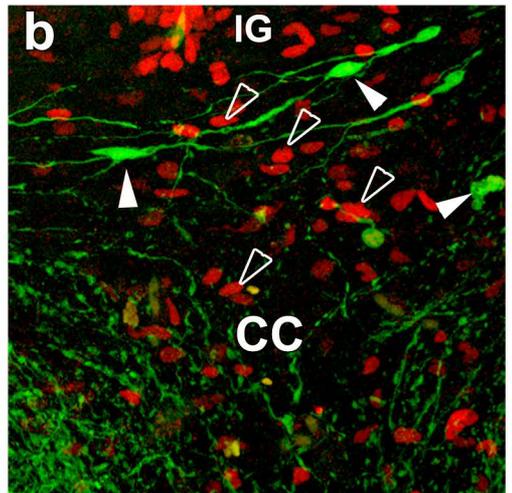
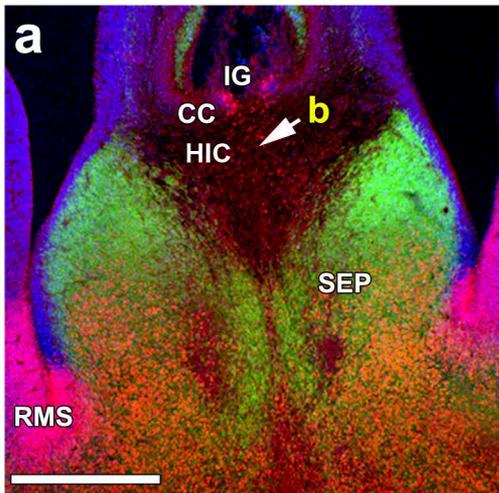
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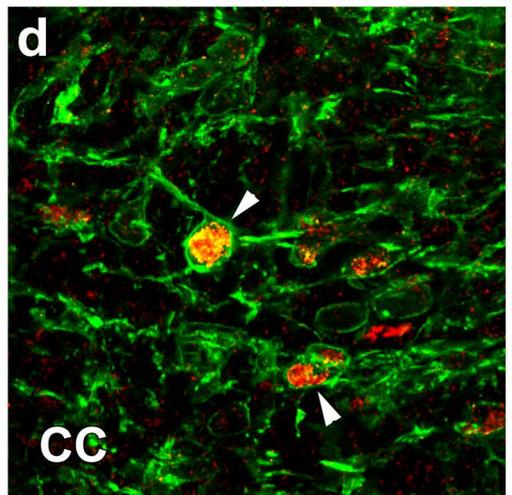
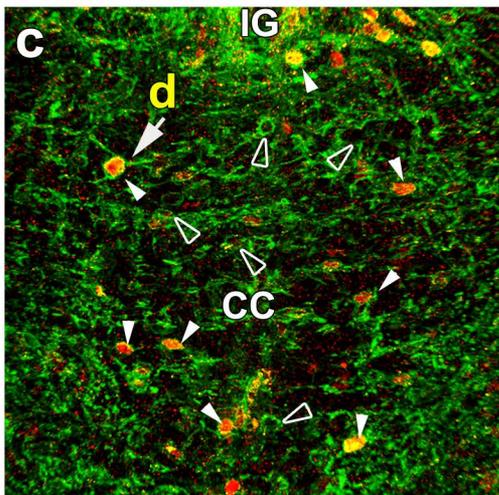
E16.5 / **Nkx2.1** / **Hoechst**

**Gad1-EGFP+**



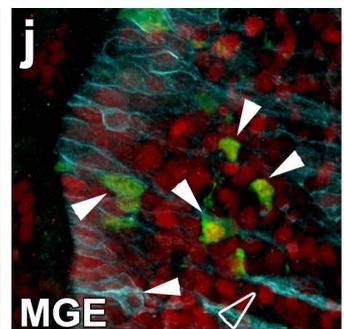
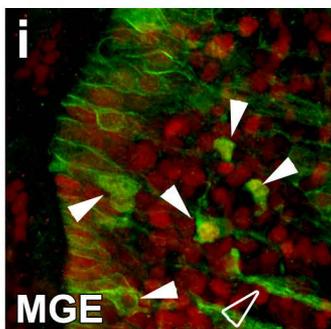
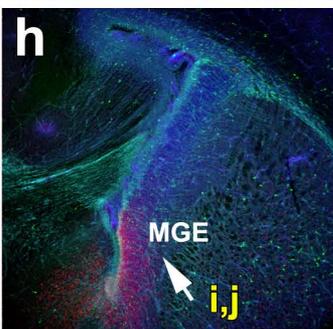
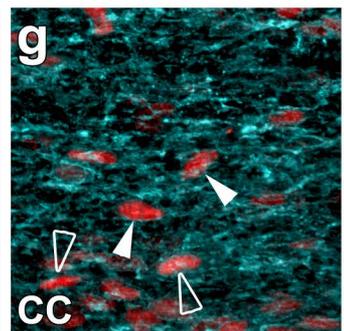
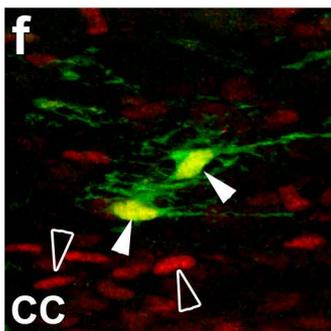
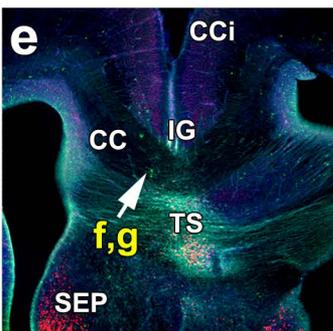
E16.5 / **GLAST** / **Nkx2.1**

**Wild-type**



E18.5 / **Nkx2.1** / **GLAST** / **Hoechst**

**GLAST-cre:ERT2+**  
**/Rosa-EYFP**



**Figure 1**

990 **Figure 1. The Nkx2.1-positive cells of the CC are glial cells.**

991 **(a-d)** Double immunohistochemistry for the GFP and Nkx2.1 **(a-b)** on coronal CC  
992 sections from *Gad1-EGFP*<sup>+</sup> mice at E16.5 (n=4), and for GLAST and Nkx2.1 (n=2)  
993 **(c-d)** on coronal CC sections from wild-type mice at E16.5. **(e-j)** Triple  
994 immunohistochemistry for the EYFP, Nkx2.1 and GLAST on coronal CC **(e-g)** and  
995 MGE **(h-j)** sections from *GLAST-Cre:ERT2*<sup>+</sup>/*Rosa-EYFP* mice at E18.5 (n=5). Cell  
996 nuclei were counterstained in blue with Hoechst **(a, e and h)**. Colocalization between  
997 the green and the red channel is highlighted in yellow **(b, c, d, f, i and j)**. **b, d, f, g, i,**  
998 **and j** are higher power views of the CC and MGE region indicated by an arrow in **a,**  
999 **c, e and h**, respectively.

1000 **(a-d)** At E16.5, several Nkx2.1<sup>+</sup> (red) nuclei were observed in the medial part of the  
1001 CC (open arrowheads in **b**). Most of the *Gad1-EGFP*<sup>+</sup> interneurons (green) populating  
1002 this region were not labelled by Nkx2.1 (solid arrowhead in **b**). At this age, however,  
1003 colocalization revealed that most of the Nkx2.1-expressing nuclei co-expressed  
1004 astroglial markers like GLAST (solid arrowheads in **c and d**). **(e-j)** The Cre-mediated  
1005 recombination was initiated under the control of the tamoxifen-inducible GLAST  
1006 promoter at E14, and the GLAST-derived astroglia were visualized (in green) with the  
1007 EYFP signal. Some GLAST<sup>+</sup> astroglial cells (in light blue) of the CC **(e-g)** and the  
1008 MGE **(i-j)** co-expressed Nkx2.1 (in red, solid arrowheads). Some of the  
1009 GLAST<sup>+</sup>/Nkx2.1<sup>+</sup> glia were not labelled by the EYFP signal and might have been  
1010 generated before the recombination was induced (open arrowheads in **f and g**). **(CC)**  
1011 corpus callosum; **(CCi)** cingulate cortex; **(IG)** induseum griseum; **(HIC)** hippocampal  
1012 commissure; **(MGE)** medial ganglionic eminence; **(RMS)** rostral migratory stream;  
1013 **(SEP)** septum; **(TS)** triangular septal nucleus. Bar = 675 μm in **e and h**; 450 μm in **a**;  
1014 67 μm in **b and c**; 40 μm in **f, g, i and j**; 30 μm in **d**.



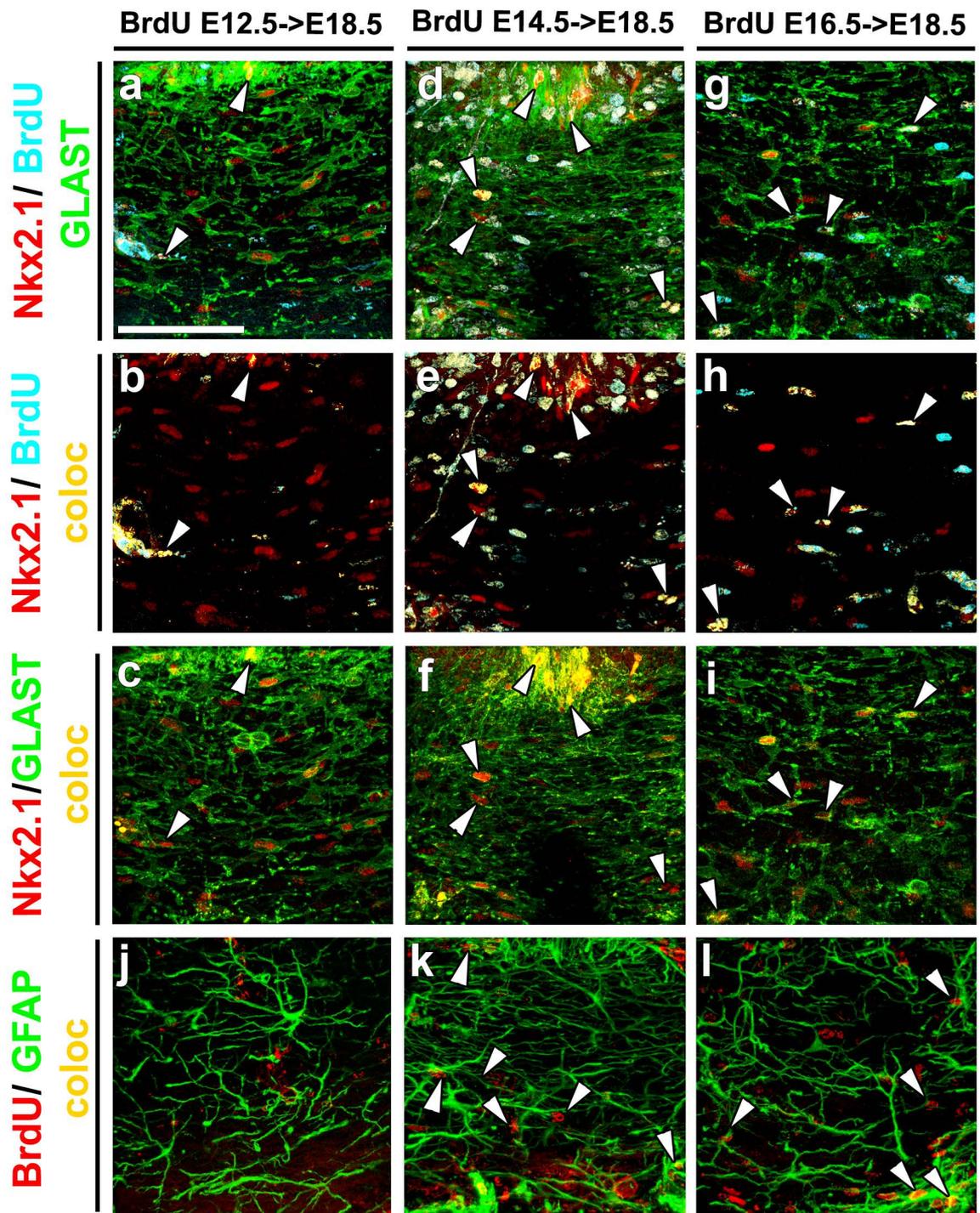
1015

1016 **Figure 2. Loss of GLAST<sup>+</sup>/Olig2<sup>-</sup> astroglia and GLAST<sup>-</sup>/Olig2<sup>+</sup> polydendrocytes**  
1017 **in the CC of *Nkx2.I*<sup>-/-</sup> mutant mice.**

1018 **(a-d)** Double immunohistochemical staining for Olig2 and GLAST on CC coronal  
1019 sections from wild-type *Nkx2.I*<sup>+/+</sup> (n=4) **(a-b)** and *Nkx2.I*<sup>-/-</sup> (n=2) **(c-d)** mice at  
1020 E18.5. Cell nuclei were counterstained in blue with Hoechst **(a-d)**. **b** and **d** are  
1021 higher power views of the CC seen in **a** and **c**, respectively. **(b** and **d)** In the CC  
1022 midline of *Nkx2.I*<sup>-/-</sup> mice, there was a severe loss of GLAST<sup>+</sup>/Olig2<sup>-</sup> astroglia  
1023 (arrows) and GLAST<sup>-</sup>/Olig2<sup>+</sup> polydendrocytes (open arrowheads) but not of  
1024 GLAST<sup>+</sup>/Olig2<sup>+</sup> astroglia (solid arrowheads) compared to the wild-type mice.

1025 **(e)** Bars (mean ± SEM from a sample of n=3 wild-type and n=3 *Nkx2.I*<sup>-/-</sup>) represent  
1026 the density (number of cells/mm<sup>3</sup>) of GLAST<sup>+</sup>/Olig2<sup>-</sup>, GLAST<sup>-</sup>/Olig2<sup>+</sup> and  
1027 GLAST<sup>+</sup>/Olig2<sup>+</sup> glial cells in the CC of *Nkx2.I*<sup>-/-</sup> (KO) compared to wild-type (WT)  
1028 mice at E18.5. The quantification confirms the significant decrease of the  
1029 GLAST<sup>+</sup>/Olig2<sup>-</sup> astroglia (p-value=0.0297) and GLAST<sup>-</sup>/Olig2<sup>+</sup> polydendrocytes (p-  
1030 value=0.0242) and no change in GLAST<sup>+</sup>/Olig2<sup>+</sup> astroglia (p-value=0.4469) in the  
1031 *Nkx2.I*<sup>-/-</sup> CC compared to WT mice. **(CC)** corpus callosum; **(CCi)** cingulate cortex;  
1032 **(CI)** cingulate bundle. Bar = 675 μm in **a** and **c**; 100 μm in **b** and **d**.

1033



**Figure 3**

1034

1035 **Figure 3. Nkx2.1-positive astroglia of the CC are generated between E14.5 and**  
1036 **E16.5.**

1037 **(a-i)** Triple immunohistochemistry for Nkx2.1, 5-bromo-2'-deoxyuridine (BrdU), and  
1038 GLAST, and **(j-l)** double immunohistochemistry for BrdU and GFAP on CC coronal  
1039 sections from wild-type mice brains at E18.5 injected at E12.5 (n=2) **(a-c and j)**,  
1040 E14.5 (n=2) **(d-f and k)** and E16.5 (n=2) **(g-i and l)**.

1041 **(a-i)** At E18.5, several GLAST<sup>+</sup> astroglial cells (green) expressing Nkx2.1 (red) are  
1042 present in the CC midline. **(b, e and h)** Colocalization between the blue (BrdU) and  
1043 the red (Nkx2.1) channel is highlighted in yellow. **(c, f and i)** Colocalization between  
1044 the green (GLAST) and the red (Nkx2.1) channel is highlighted in yellow. The solid  
1045 arrowheads point towards the Nkx2.1<sup>+</sup>/GLAST<sup>+</sup>/BrdU<sup>+</sup> cells revealing that the bulk of  
1046 division for the Nkx2.1<sup>+</sup> astroglial cells of the CC occurs between E14.5 **(e)** and  
1047 E16.5 **(h)**.

1048 **(j-l)** Numerous GFAP<sup>+</sup> astroglial cells (in green) are present in the CC midline.  
1049 Colocalization between the green (GFAP) and the red (BrdU) channel is highlighted  
1050 in yellow. The solid arrowheads are pointing on the GFAP<sup>+</sup>/BrdU<sup>+</sup> cells depicting that  
1051 the bulk of division for the GFAP<sup>+</sup> glial cells of the CC occurs also from E14.5 **(k)** to  
1052 E16.5 **(l)**. Bar = 60 μm in **a-l**.

1053

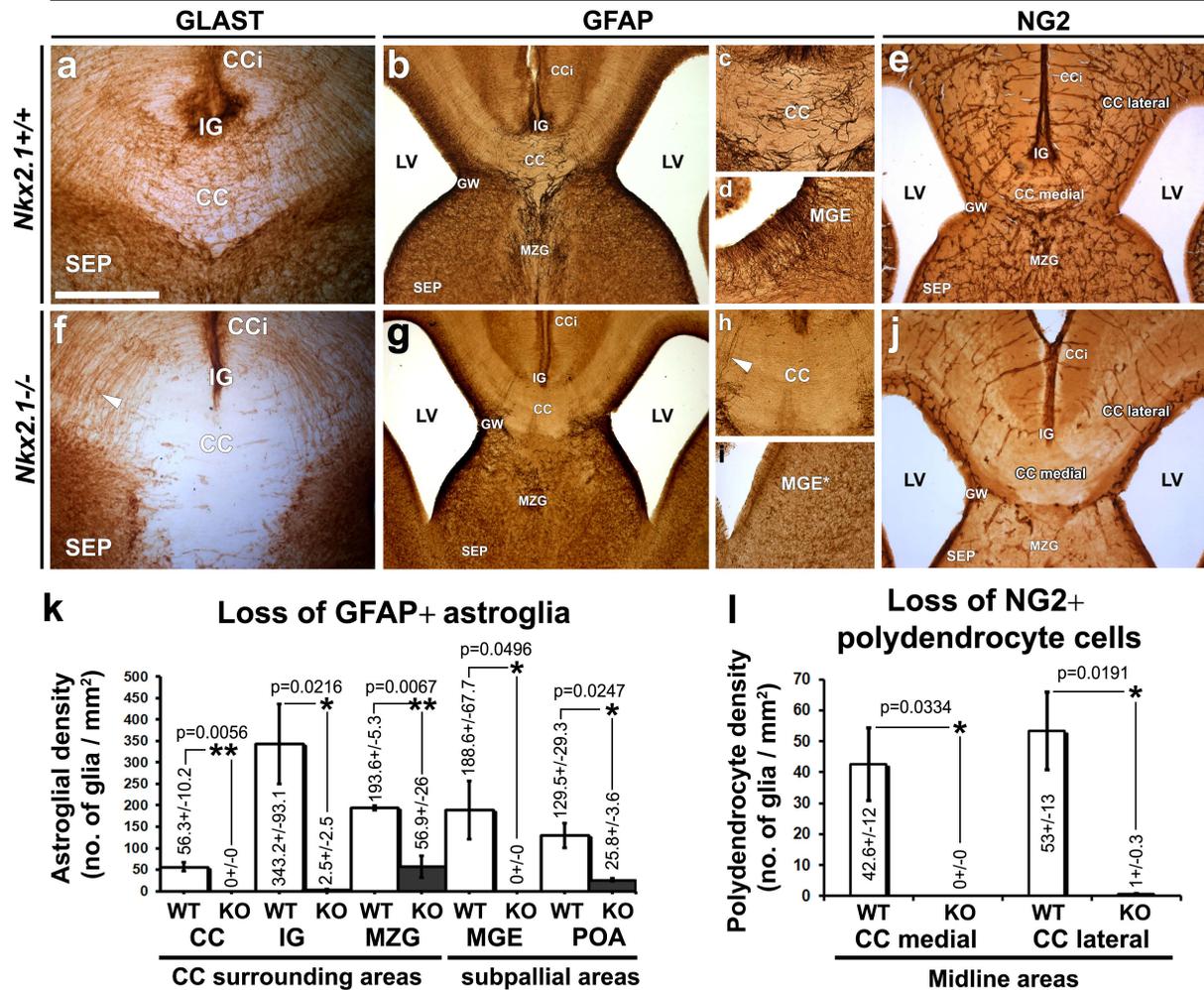


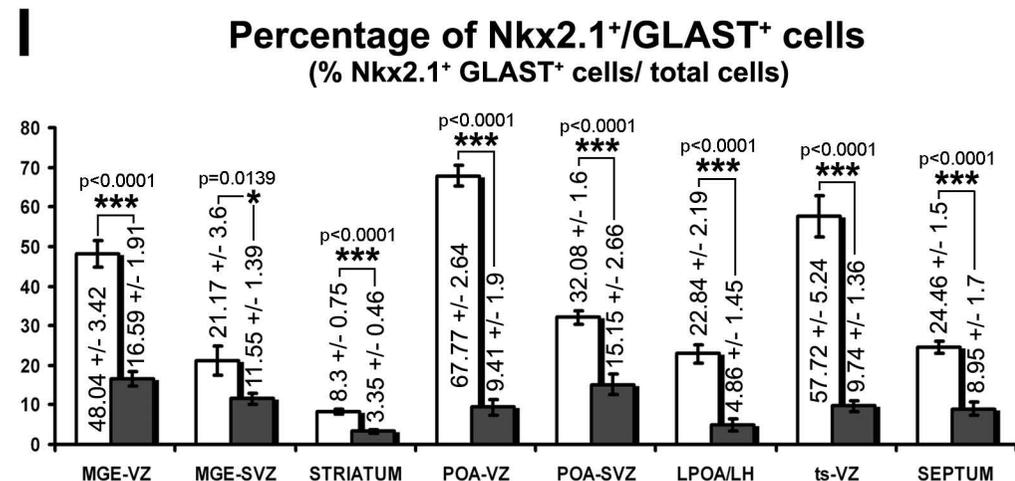
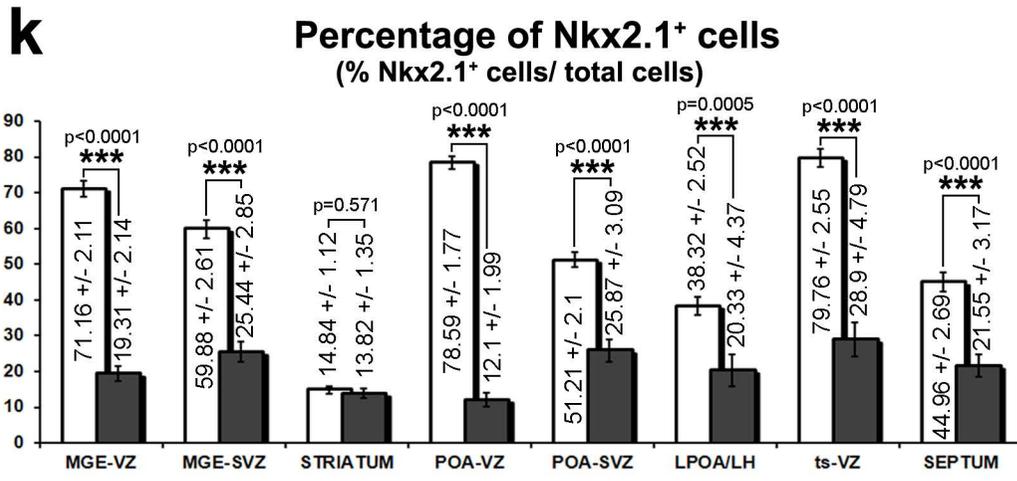
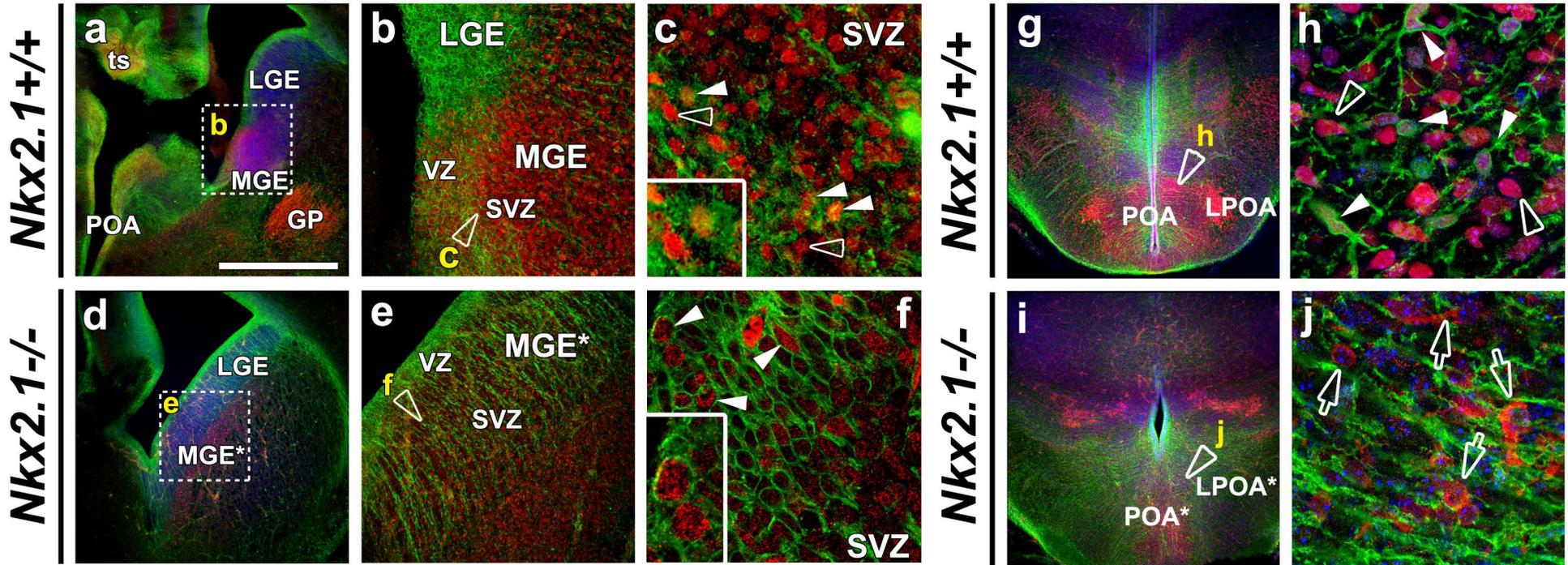
Figure 4

1054

1055 **Figure 4. Loss of different glial cell types in the CC, medial cortical areas and**  
1056 **subpallium of *Nkx2.1*<sup>-/-</sup> mice brains.**

1057 DAB staining for GLAST (n=2 for WT and *Nkx2.1*<sup>-/-</sup>) (**a** and **f**), GFAP (n=4 for WT  
1058 and 3 for *Nkx2.1*<sup>-/-</sup>) (**b-d** and **g-i**) and NG2 (n=5 for WT and n=4 for *Nkx2.1*<sup>-/-</sup>) (**e** and  
1059 **j**) on CC and MGE coronal sections from wild-type (**a-e**) and *Nkx2.1*<sup>-/-</sup> (**f-j**) mice at  
1060 E18.5. **c** and **h** are higher power views of the CC region seen in **b** and **g**,  
1061 respectively. **d** and **i** are higher power views of the MGE region. DAB staining for  
1062 GLAST, GFAP and NG2 revealed a drastic loss of astroglial and polydendroglial  
1063 cell types from the CC and surrounding areas and from the MGE of the *Nkx2.1*<sup>-/-</sup>  
1064 mice compared to wild-type mice (compare **f** with **a**, **g-h** with **b-c**, **i** with **d** and **j**  
1065 with **e**). Only the GFAP<sup>+</sup> radial glial cells originating from the *Nkx2.1*<sup>-</sup> glial wedge  
1066 (GW) bordering the CC remained unaffected (white arrowhead in **f** and **h**). (**k** and **l**)  
1067 Bars (mean ± SEM from a sample of n=4 brains in WT and n=3 brains in *Nkx2.1*<sup>-/-</sup>  
1068 for GFAP and n=5 in WT and n= 3 in *Nkx2.1*<sup>-/-</sup> for NG2) represent the cell densities  
1069 of GFAP<sup>+</sup> or NG2<sup>+</sup> glial cells/mm<sup>2</sup>. The quantification of the GFAP<sup>+</sup> (p-value=0.056  
1070 for CC, 0.0216 for IG, 0.0067 for MZG, 0.0496 for MGE, and 0.0247 for POA) and  
1071 NG2<sup>+</sup> (p-value=0.0334 for CC medial and 0.0191 for CC lateral) glial cell density  
1072 showed a drastic and significant loss of these cells in the CC and surrounding areas  
1073 as well as in medial cortical areas of the *Nkx2.1*<sup>-/-</sup> brain compared to the wild-type  
1074 brains. (**CC**) corpus callosum; (**CCi**) cingulate cortex; (**GW**) glial wedge; (**IG**)  
1075 induseum griseum; (**LV**) lateral ventricle; (**MZG**) midline zipper glia; (**MGE**)  
1076 medial ganglionic eminence; (**SEP**) septum. Bar = 500 μm in **b**, **e**, **g** and **j**; 250 μm  
1077 in **a**, **f**, **c**, **d**, **h** and **i**.

# E16.5 / **Nkx2.1** / **GLAST** / **Hoechst**



□ Nkx2.1<sup>+/+</sup>    ■ Nkx2.1<sup>-/-</sup>

Telencephalic regions

Figure 5

1078

1079 **Figure 5. Incapacity of precursors to generate astrocytes in *Nkx2.1*<sup>-/-</sup> mice**  
1080 **brains.**

1081 **(a-c and g-h)** Double immunohistochemical staining for Nkx2.1 and GLAST on  
1082 MGE **(a-c)** and POA **(g-h)** coronal sections from wild-type (n=4) mice brains at  
1083 E16.5. **(d-f and i-j)** Double immunohistochemical staining for mutated Nkx2.1 (mut-  
1084 Nkx2.1) and GLAST on MGE\* **(d-f)** and POA\* **(i-j)** coronal sections from *Nkx2.1*<sup>-/-</sup>  
1085 (n=4) mice brains at E16.5. Cell nuclei were counterstained in blue with Hoechst **(a,**  
1086 **d, g-h and i-j)**. **b, c, e, f, h** and **j** are higher power views of the regions seen in **a, d,**  
1087 **g** and **i** respectively.

1088 **(a-c)** In the germinal regions of the wild-type MGE, numerous Nkx2.1<sup>+</sup> progenitors  
1089 were GLAST<sup>+</sup> (solid arrowheads and inset in **c**), while some other were GLAST<sup>-</sup>  
1090 (open arrowheads in **c**). **(d-f)** In *Nkx2.1*<sup>-/-</sup> MGE\* germinal regions, only few  
1091 GLAST<sup>+</sup> progenitors expressed the mutated Nkx2.1 protein (solid arrowheads and  
1092 inset in **f**).

1093 **(g-h)** In the parenchyma of wild-type POA, many GLAST<sup>+</sup> astroglial cells (solid  
1094 arrowheads in **h**) and neurons expressed Nkx2.1. **(i-j)** In the parenchyma of *Nkx2.1*<sup>-/-</sup>  
1095 POA\*, GLAST<sup>+</sup> astroglial cells have disappeared and only few neurons expressing  
1096 the mutated Nkx2.1 protein are observed (open arrows in **j**).

1097 **(GP)** globus pallidus; **(LGE)** lateral ganglionic eminence; **(LPOA)** lateral POA;  
1098 **(MGE)** medial ganglionic eminence; **(MGE\*)** mutant medial ganglionic eminence;  
1099 **(POA)** preoptic area; **(POA\*)** mutant preoptic area; **(SEP)** septum; **(SVZ)**

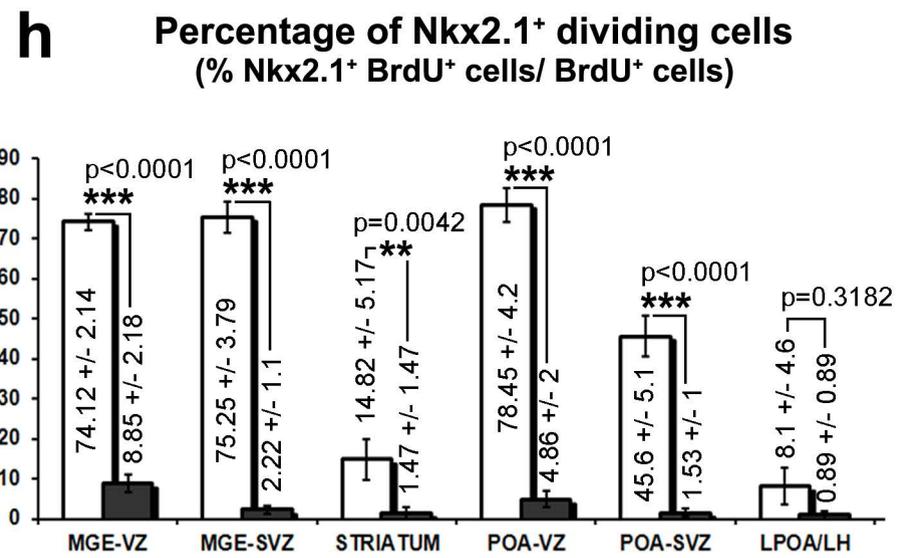
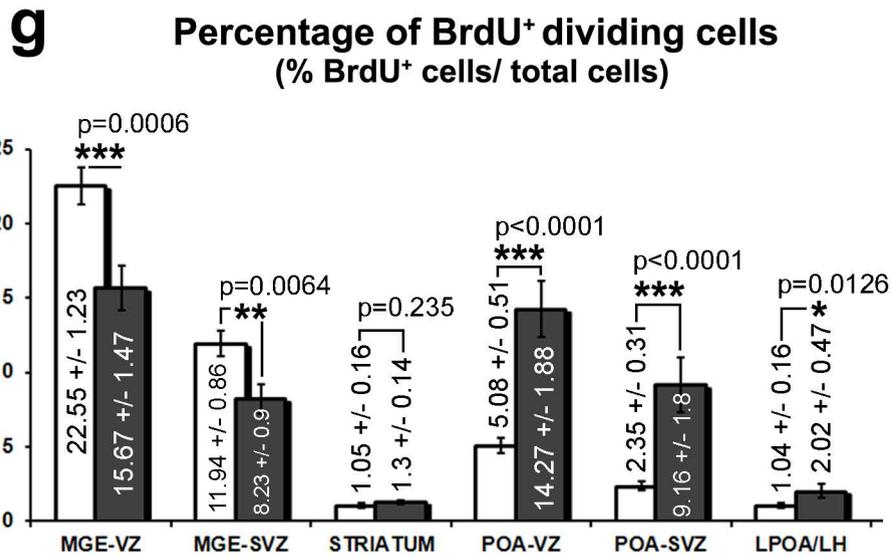
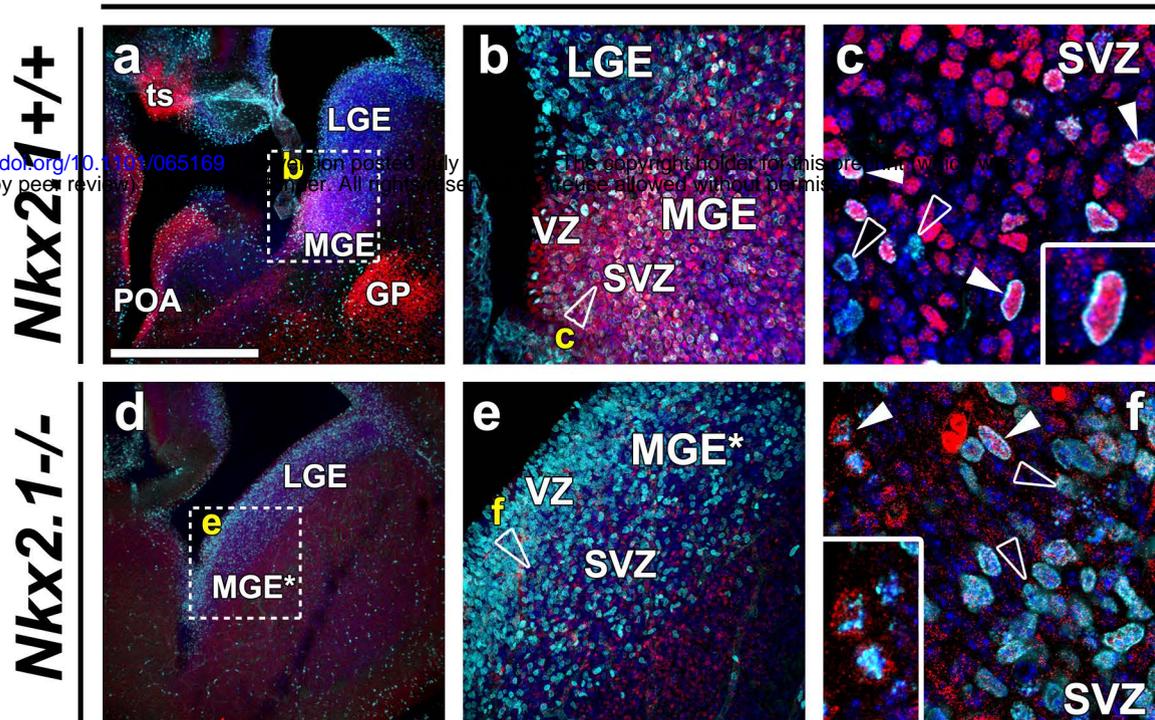
1100 subventricular zone; **(TS)** triangular septal nucleus, **(VZ)** ventricular zone. Bar: 100  
1101  $\mu\text{m}$  in **b** and **e**; 45  $\mu\text{m}$  in **c** and **f** and 50  $\mu\text{m}$  in **h** and **j**.

1102 **(k-l)** Bars (mean  $\pm$  SEM from n=4 brains in WT and n=4 brains in  $Nkx2.1^{-/-}$  mice)  
1103 represent the percentage of  $Nkx2.1^{+}$  cells **(k)** and of  $Nkx2.1^{+}/GLAST^{+}$  precursors  
1104 and astroglial cells **(l)** in WT (white columns) and  $Nkx2.1^{-/-}$  (black columns)  
1105 subpallial germinal (MGE, POA and TS: VZ and SVZ) and parenchymal (striatum,  
1106 LPOA/LH, septum) telencephalic regions at E16.5. **(k and l)** The number of cells **(k)**  
1107 and  $GLAST^{+}$  **(l)** precursors and post-mitotic cells expressing the mutated  $Nkx2.1$   
1108 was drastically decreased in all the subpallial telencephalic regions of the  $Nkx2.1^{-/-}$ .  
1109 The p-values are indicated above the respective graphs within the figure panels k to  
1110 l.

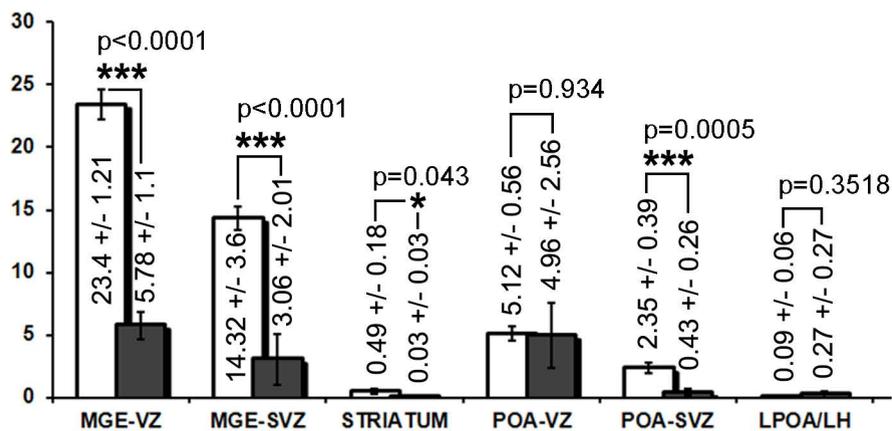
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E16.5 / **Nkx2.1** / **BrdU** / **Hoechst**

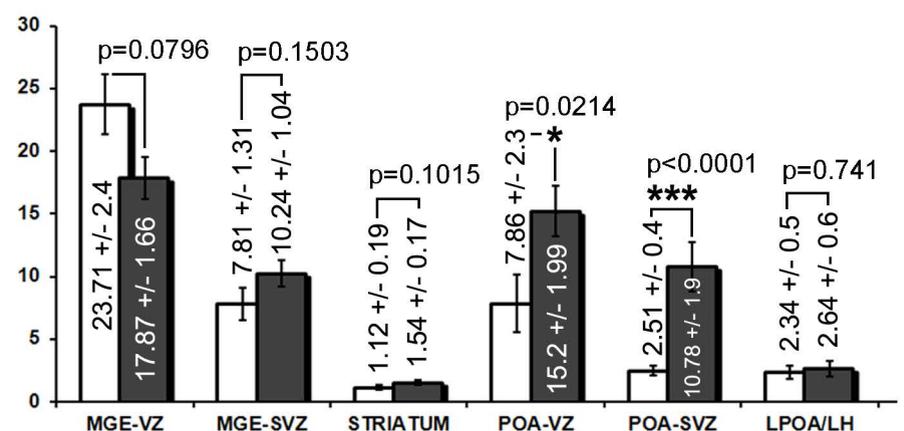
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**i** Within Nkx2.1<sup>+</sup> cells, how many cells divide? (% Nkx2.1<sup>+</sup> BrdU<sup>+</sup> cells/ Nkx2.1<sup>+</sup> cells)



**j** Within Nkx2.1<sup>-</sup> cells, how many cells divide? (% Nkx2.1<sup>-</sup> BrdU<sup>+</sup> cells/ Nkx2.1<sup>-</sup> cells)



□ *Nkx2.1*<sup>+/+</sup>      ■ *Nkx2.1*<sup>-/-</sup>

Telencephalic regions

Figure 6

1112

1113 **Figure 6. Incapacity of Nkx2.1<sup>+</sup> precursors to divide after Nkx2.1 inactivation.**

1114 **(a-c)** Double immunohistochemical staining for Nkx2.1 and 5-bromo-2'-  
1115 deoxyuridine (BrdU) on telencephalic coronal sections from wild-type (n=4) mice  
1116 brains at E16.5. **(d-f)** Double immunohistochemical staining for mutated Nkx2.1 and  
1117 BrdU on telencephalic coronal sections from *Nkx2.1*<sup>-/-</sup> (n=4) mice brains at E16.5.  
1118 Cell nuclei were counterstained in blue with Hoechst **(a-f)**. **b** and **e** are higher power  
1119 views of the MGE squared regions seen in **a** and **d**. **c** and **f** are higher magnifications  
1120 of the MGE seen in **b** and **e**, respectively. **(a-c)** In the VZ and the SVZ of the wild-  
1121 type MGE, AEP/POA and TS, numerous Nkx2.1<sup>+</sup> precursors (in red) co-labelled for  
1122 BrdU (in light blue) were dividing at E16.5 (solid arrowheads and inset in **c**). Other  
1123 BrdU<sup>+</sup> dividing cells were not labelled by Nkx2.1 (open arrowheads in **c**). **(d-f)** In  
1124 the VZ and the SVZ of the *Nkx2.1*<sup>-/-</sup> MGE (MGE\*), numerous precursors co-labelled  
1125 by the BrdU were also seen to divide (open arrowheads in **f**), but only few dividing  
1126 cells were expressing the mutated Nkx2.1 protein (solid arrowheads and inset in **f**).

1127 **(LGE)** lateral ganglionic eminence; **(MGE)** medial ganglionic eminence; **(MGE\*)**  
1128 mutant medial ganglionic eminence; **(POA)** preoptic area; **(SVZ)** subventricular  
1129 zone; **(TS)** triangular septal nucleus, **(VZ)** ventricular zone. Bar = 675 μm in **a** and  
1130 **d**; 100 μm in **b** and **c** and 45 μm in **e** and **f**.

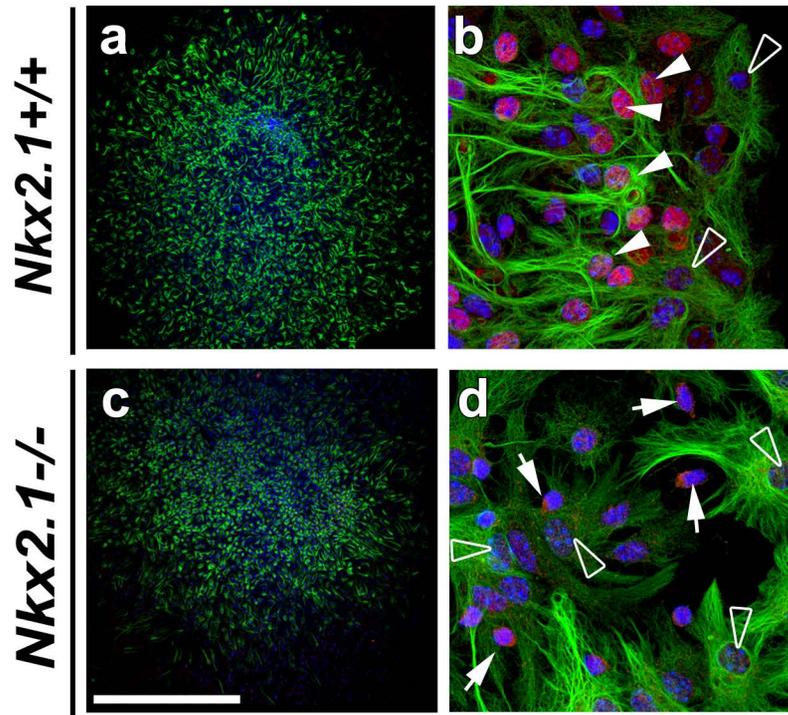
1131 **(g-j)** Bars (mean ± SEM from n=4 brains from WT and n=4 brains from *Nkx2.1*<sup>-/-</sup>  
1132 mice) represent the percentage of the BrdU<sup>+</sup> dividing cells **(g)**; the percentage of  
1133 BrdU<sup>+</sup> dividing cells which are also positive for Nkx2.1 or mutated Nkx2.1 **(h)**; the  
1134 percentage of cells for Nkx2.1 or mutated Nkx2.1 that divided **(i)**, and the percentage  
1135 of Nkx2.1<sup>-</sup> cells that divided **(j)** in WT and *Nkx2.1*<sup>-/-</sup> germinal (MGE, POA and TS:

1136 VZ and SVZ) and parenchymal (striatum, LPOA/LH) telencephalic regions at E16.5.  
1137 **(g)** A significant decrease of the BrdU<sup>+</sup> dividing precursors in the VZ and SVZ of  
1138 the MGE\* was balanced by a significant increase of BrdU<sup>+</sup> dividing precursors in  
1139 the VZ, SVZ of the POA\* of Nkx2.1<sup>-/-</sup>; **(h)** a drastic and significant decrease in the  
1140 dividing cells which expressed mutated Nkx2.1 was observed in all regions of  
1141 Nkx2.1<sup>-/-</sup>; **(i)** the cells that express the mutated Nkx2.1, lost their capacity to divide  
1142 in the VZ and SVZ of the MGE\*, the striatum and the SVZ of the POA\*; **(j)** by  
1143 contrast, the cells that do not express mutated Nkx2.1, still divided normally in the  
1144 MGE\* and maintained the capacity to divide in the VZ and SVZ of the POA\* of  
1145 Nkx2.1<sup>-/-</sup>. The p-values are indicated above the respective graphs within the figure  
1146 panels g to j.

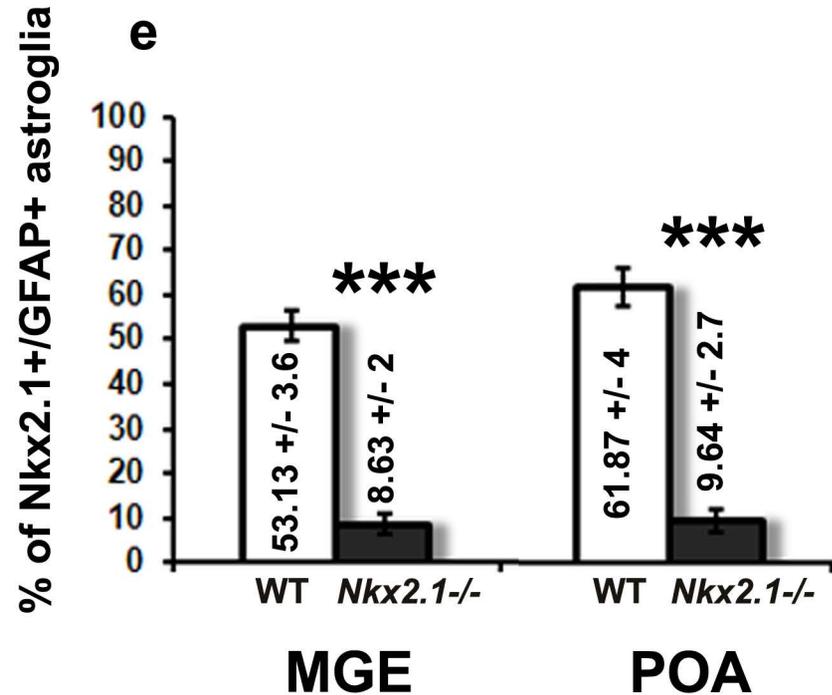
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# MGE Neurospheres at DIV7

Hoechst / Nkx2.1 / GFAP



## Percentage of Nkx2.1+/GFAP+ astroglia in MGE and POA-derived neurospheres



# Figure 7

1148

1149 **Figure 7. Nkx2.1<sup>+</sup> MGE and POA stem cells do not differentiate into astrocytes**  
1150 **after *Nkx2.1* inactivation.**

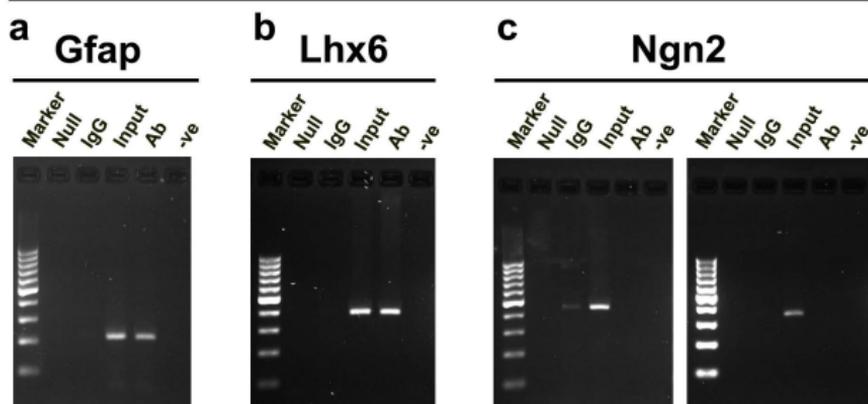
1151 **(a-b)** Double immunocytochemistry for Nkx2.1 and GFAP on MGE-derived  
1152 neurospheres from E14.5 wild-type mice (n=8) after 7 days *in vitro* (DIV). **(c-d)**  
1153 Double immunocytochemistry for mutated Nkx2.1 and GFAP on MGE\*-derived  
1154 neurospheres from E14.5 *Nkx2.1*<sup>-/-</sup> mice (n=6) after 7 days *in vitro* (DIV). Cell nuclei  
1155 were counterstained in blue with Hoechst **(a-d)**. **b** and **d** are higher power views of  
1156 the regions seen in **a** and **c** respectively. In neurospheres derived from wild-type  
1157 MGE, numerous GFAP<sup>+</sup> astrocytes were labelled for Nkx2.1 (solid arrowheads in  
1158 **b**), while some others were not (open arrowheads in **b**). By contrast, in neurospheres  
1159 derived from *Nkx2.1*<sup>-/-</sup> MGE\*, the GFAP<sup>+</sup> astrocytes were never observed to be co-  
1160 labelled for the mutated Nkx2.1 (open arrowheads in **d**), but neuronal cells still  
1161 express low levels of the mutated Nkx2.1 protein (arrows in **d**). Bar = 675 μm in **a**  
1162 and **c** and 50 μm in **b** and **d**.

1163 **(e)** Bars (mean ± SEM from a sample of n=21 MGE, n=34 POA WT neurospheres  
1164 and n=22 MGE\*, n=28 POA\* *Nkx2.1*<sup>-/-</sup> neurospheres) represent the percentage of  
1165 GFAP<sup>+</sup> astrocytes labeled for Nkx2.1 in MGE- or POA-derived neurospheres from  
1166 wild-type and of GFAP<sup>+</sup> astrocytes labeled for mutated Nkx2.1 in MGE\*- or POA\*-  
1167 derived neurospheres from *Nkx2.1*<sup>-/-</sup> mice brains. Neurospheres originating from  
1168 *Nkx2.1*<sup>-/-</sup> MGE\* and POA\* nearly lost the capacity to produce Nkx2.1-derived  
1169 astrocytes (p-value<0.0001 for both).

1170

# Chromatin Immunoprecipitation on E16.5 brains

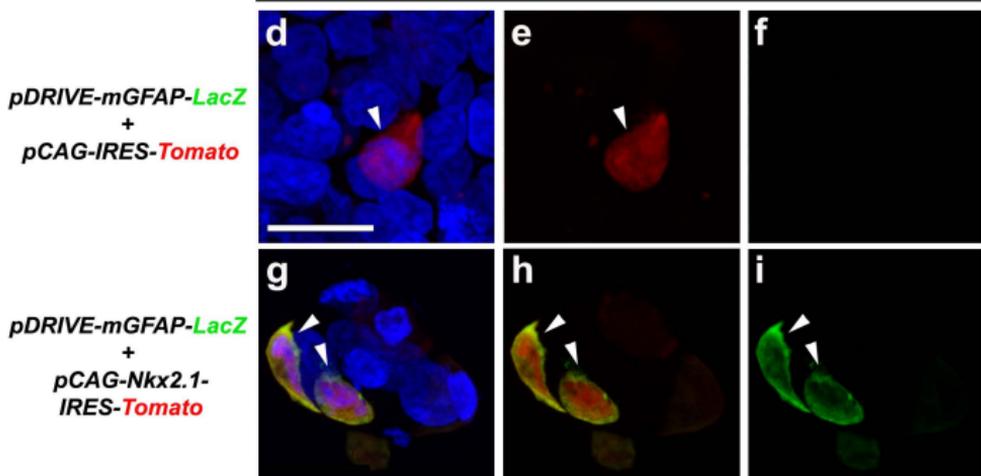
Nkx2.1 binding to conserved consensus sequences in the promoter region of various regulatory genes



## Transfection of HEK293 cells

Nkx2.1 activates the expression of GFAP promoter reporter

LacZ / Tomato / Hoechst



1171 **Figure 8. Binding of Nkx2.1 to conserved binding sequences in the promoters of**  
1172 **various glial regulatory genes.**

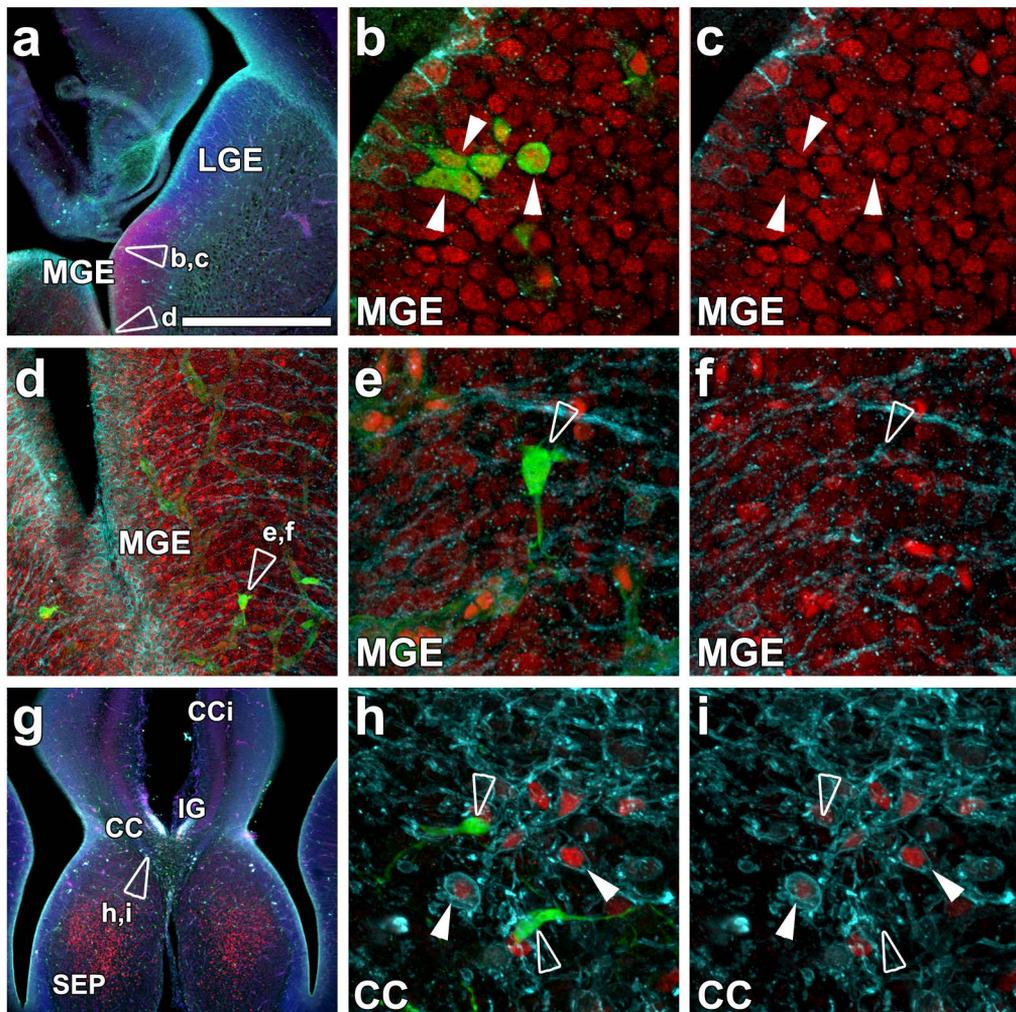
1173 Amplification of the Nkx2.1 putative binding sequences located in a 206 bp PCR  
1174 product within the GFAP promoter (**a**) and in a 391 bp PCR fragment within the Lhx6  
1175 promoter (**b**) after chromatin immunoprecipitation with the Nkx2.1 antibody. Input  
1176 DNA was added as the positive loading control as it contains the crosslinked  
1177 sonicated genomic DNA taken before chromatin immunoprecipitation with the  
1178 Nkx2.1 antibody, and a strong signal was observed for all the promoter regions. No  
1179 amplification of the Nkx2.1 core sequence (tcaag) located in two, 410 bp and 352 bp,  
1180 PCR products within the Ngn2 promoter was detected (**c**). No signal was detected in  
1181 the null control, wherein no antibody was added for chromatin immunoprecipitation  
1182 and in the negative control, wherein no DNA was added while performing the PCR  
1183 (**a-c**). A very faint signal was detected in some of the samples immunoprecipitated  
1184 with non-specific control IgG but its intensity was much lower than the intensity of  
1185 the input DNA and the test DNA (containing promoter region). These results suggest  
1186 that Nkx2.1 binds the promoter regions of various glial regulatory genes at a  
1187 conserved Nkx2.1 binding sequence *in vivo*. The figure represents one of the three  
1188 independently performed assays. Identical results were also obtained for the same  
1189 glial regulatory genes in the E14.5 brain samples.

1190 As a control, the human embryonic kidney 293 (HEK293) cells were co-transfected  
1191 with 2.0 µg of two reporter constructs, namely, the *pDRIVE-mGFAP-LacZ* expression  
1192 plasmid containing the LacZ reporter under the control of the mouse 1679 bp  
1193 upstream GFAP promoter sequence, and the *pCAG-IRES-Tomato* plasmid  
1194 constitutively expressing the Tomato reporter under the control of the pCAG  
1195 promoter (**d-f**). To test the binding of the Nkx2.1 to the GFAP promoter sequence, the

1196 HEK293 cells were co-transfected with 2.0  $\mu$ g of two reporter constructs, namely, the  
1197 *pDRIVE-mGFAP-LacZ* expression plasmid, and the *pCAG-Nkx2.1-IRES-Tomato*  
1198 plasmid expressing Nkx2.1 protein tagged with Tomato under the control of the  
1199 constitutive promoter pCAG (**g-i**). Cell nuclei were counterstained in blue with  
1200 Hoechst. Activation of the LacZ reporter was seen upon addition of the Nkx2.1  
1201 expression vector, thus confirming that Nkx2.1 activates the GFAP promoter, most  
1202 probably through the binding sequence that we identified. Bar = 50  $\mu$ m.  
1203

E16.5 / **Hoechst** / **Nkx2.1** / **GLAST**

*Cspg4-cre+/Rosa-EYFP*



**Supplementary Figure S1**

1204

1205 **Figure 1-figure supplement 1. Fate-mapping study of *Nkx2.1*-regulated NG2<sup>+</sup>**  
1206 **polydendrocytes using the *Cspg4-Cre<sup>+</sup>/Rosa-EYFP* reporter mice.**

1207 **(a-i)** Triple immunohistochemistry for the EYFP, *Nkx2.1* and GLAST on coronal  
1208 sections from *Cspg4-Cre<sup>+</sup>/Rosa-EYFP* mice (n=3) at E16.5. Cell nuclei were  
1209 counterstained in blue with Hoechst (**a** and **g**). **b, c, e, f, h** and **i** are higher power  
1210 views of the regions shown in **a, d** and **g**, respectively. At E16.5, NG2<sup>+</sup> (or *Cspg4<sup>+</sup>*)  
1211 polydendrocytes visualized by the EYFP signal were found to originate from *Nkx2.1<sup>+</sup>*  
1212 subpallial sites such as the MGE (**a-c** and **d-f**). The colocalization between *Nkx2.1* (in  
1213 red) and the EYFP signal (in green) is observed in few cells in the SVZ of the MGE  
1214 (solid arrowheads in **b** and **c**) but as soon as the NG2<sup>+</sup> cells start to differentiate and  
1215 migrate, *Nkx2.1* is down-regulated and is no anymore more visible (open arrowheads  
1216 in **e-f** and **h-i**). By contrast, *Nkx2.1* is still expressed in GLAST<sup>+</sup> astroglial cells  
1217 within the CC midline (solid arrowheads in **h-i**).

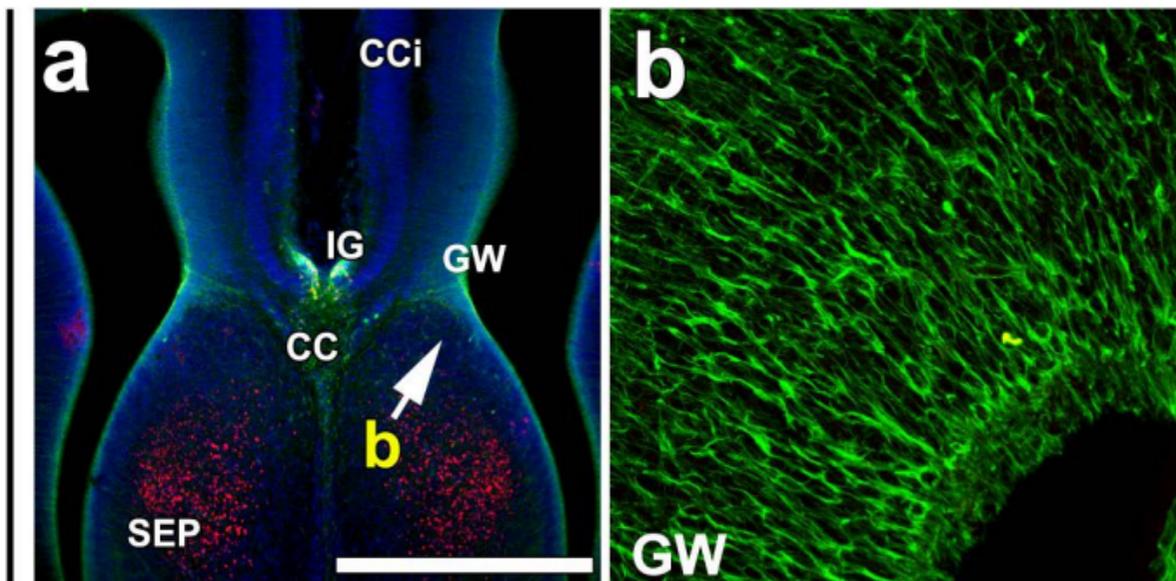
1218 **(CC)** corpus callosum; **(CCi)** cingulate cortex; **(CI)** cingulate bundle; **(IG)** induseum  
1219 griseum; **(MGE)** medial ganglionic eminence; **(SEP)** septum.

1220 Bar = 675  $\mu$ m in **a** and **g**; 160  $\mu$ m in **d**, 40  $\mu$ m in **b, c, e, f, h** and **i**.

1221

E16.5 / **Nkx2.1** / **GLAST** / **Hoechst**

**Wild-type**



**Supplementary Figure S2**

1222

1223 **Figure 1-figure supplement 2. Radial glial cells within glial wedge region are**

1224 **Nkx2.1-negative.**

1225 **(a-b)** Double immunostaining for the Nkx2.1 and GLAST on coronal sections from

1226 WT mice (n=3) at E16.5. Cell nuclei were counterstained in blue with Hoechst **(a)**. **b**

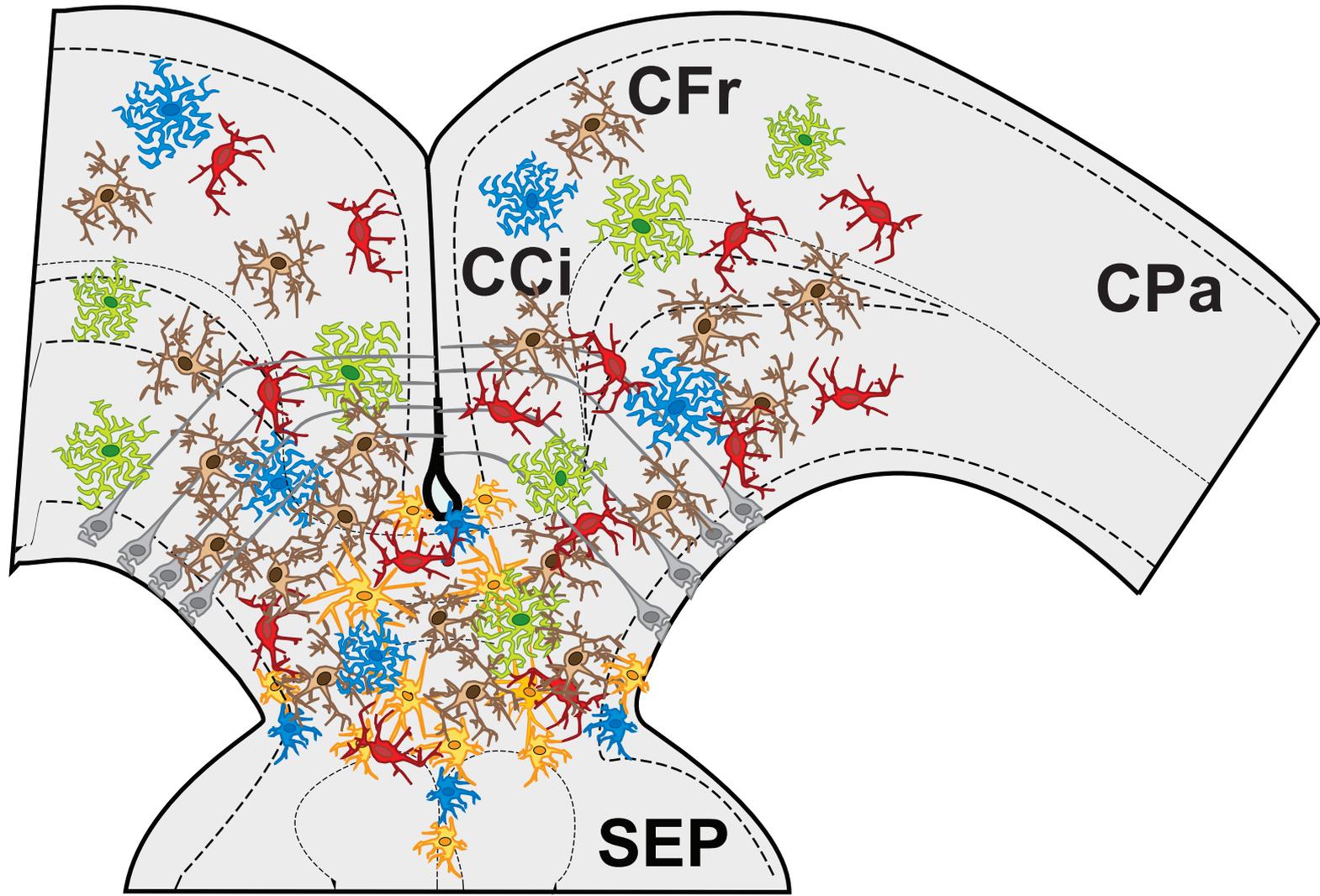
1227 is higher power view of the regions shown in **a**. The GLAST<sup>+</sup> radial glial cells within

1228 glial wedge **(GW)** do not express Nkx2.1. **(CC)** corpus callosum; **(CCi)** cingulate

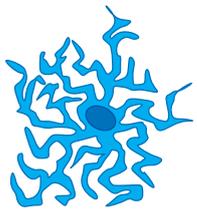
1229 cortex; **(IG)** induseum griseum; **(SEP)** septum.

1230 Bar = 675  $\mu\text{m}$  in **a** and 40  $\mu\text{m}$  in **b**.

1231



**NOT  
Nkx2.1-derived**

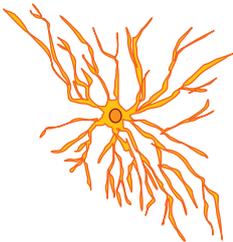


GLAST+  
GFAP-  
S100β-  
NG2-  
Olig2+  
Nkx2.1-

Not Nkx2.1-derived

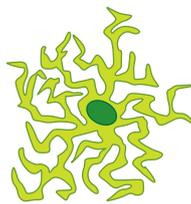
**Nkx2.1-derived**

**Astrocyte-like**



GLAST+  
GFAP+  
S100β-  
NG2-  
Olig2-  
Nkx2.1+

Nkx2.1-derived cells +



GLAST+  
GFAP-  
S100β-  
NG2-  
Olig2-  
Nkx2.1+

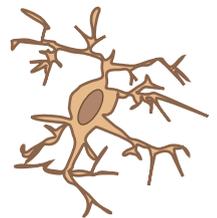
Nkx2.1-derived cells +

**Polydendrocyte-like**



GLAST-  
GFAP-  
S100β+  
NG2+  
Olig2+  
Nkx2.1-

Nkx2.1-derived cells +



GLAST-  
GFAP-  
S100β-  
NG2+  
Olig2+  
Nkx2.1-

Nkx2.1-derived cells +

**Supplementary Figure S3**

1232

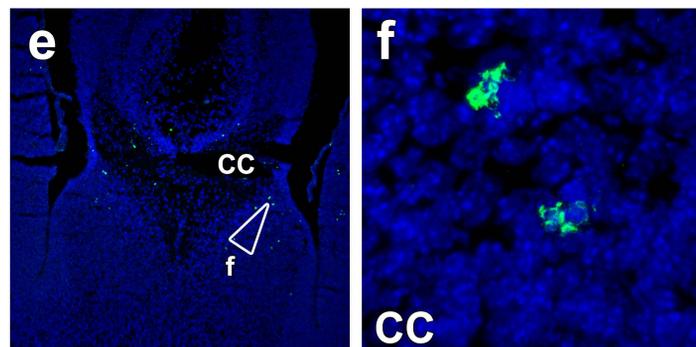
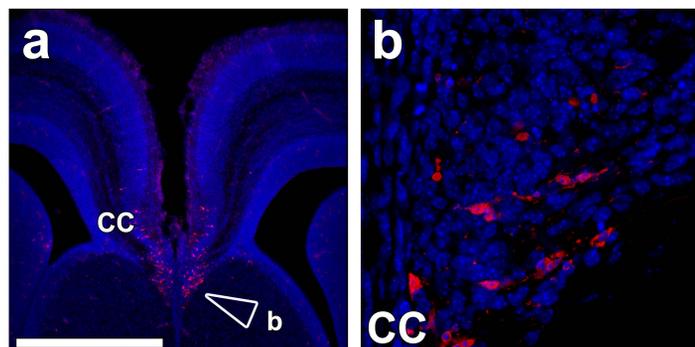
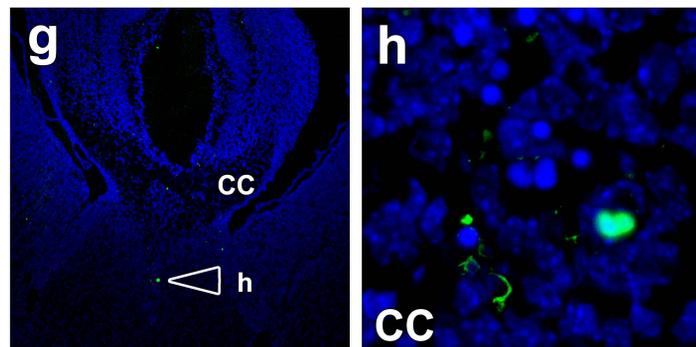
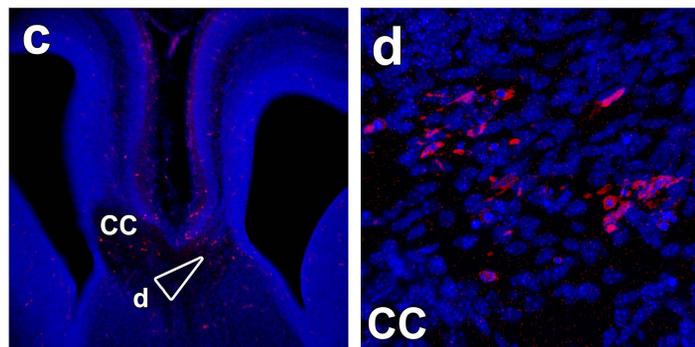
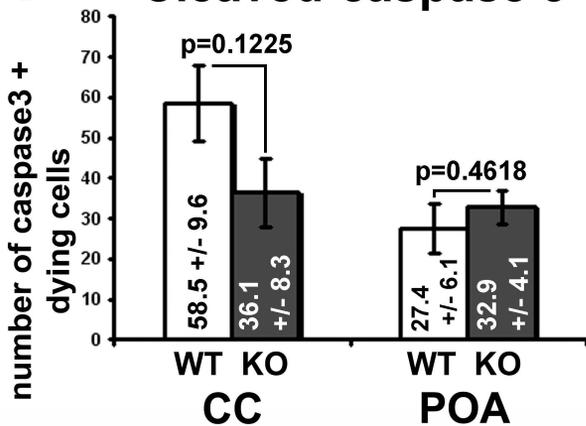
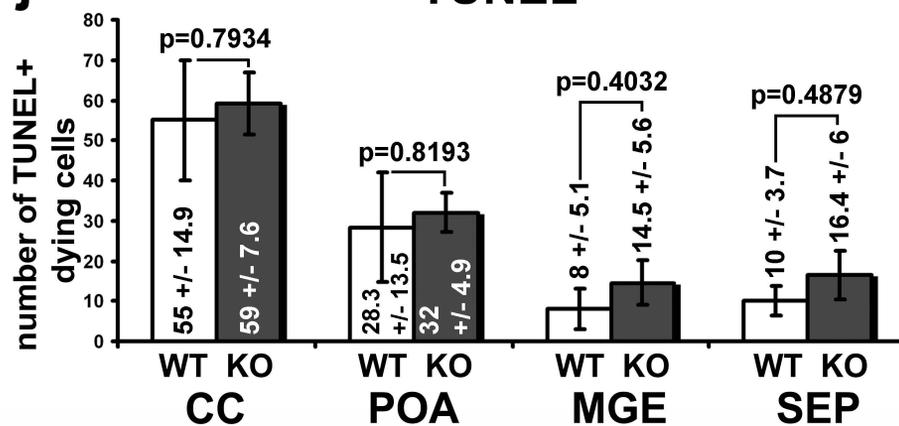
1233 **Figure 1-figure supplement 3. Four subtypes of *Nkx2.1*-derived glial cells.**

1234 The schema represents a coronal view of the CC at E18.5, and summarizes the  
1235 different types of *Nkx2.1*-derived glial populations visualized in our experiments.  
1236 The CC forms a complex environment composed of one non-*Nkx2.1*-derived glial  
1237 cell subtype and four different subtypes of *Nkx2.1*-derived glial cells. Three types of  
1238 astrocyte-like cell populations are shown: in orange, the GLAST<sup>+</sup>/GFAP<sup>+</sup>/Olig2<sup>-</sup>  
1239 /Nkx2.1<sup>+</sup> cells, in green, the GLAST<sup>+</sup>/GFAP<sup>-</sup>/Olig2<sup>-</sup>/Nkx2.1<sup>+</sup> cells, and in blue, the  
1240 GLAST<sup>+</sup>/Olig2<sup>+</sup>/Nkx2.1<sup>-</sup> cells. Two types of polydendrocyte-like cells are shown; in  
1241 red, the GLAST<sup>-</sup>/S100β<sup>+</sup>/NG2<sup>+</sup>/Olig2<sup>+</sup>/Nkx2.1<sup>-</sup> cells, and in brown, the GLAST<sup>-</sup>  
1242 /NG2<sup>+</sup>/Olig2<sup>+</sup>/Nkx2.1<sup>-</sup> cells. Under each glial cell-type category, the expression  
1243 profile of the different glial markers, employed to identify and characterize the glial  
1244 cells, used in combination with the *Nkx2.1* antibody, is presented. The (+) sign  
1245 indicates that the glial cell type was positively labelled by the listed marker, whereas  
1246 the (-) sign indicates that the glial cell type was not labelled by the listed marker.  
1247 **(CCi)** cingulate cortex, **(CFr)** frontal cortex, **(CPa)** parietal cortex, **(SEP)** septum.

1248

E16.5 / Hoechst / caspase3

E16.5 / Hoechst / TUNEL

*Nkx2.1*<sup>+/+</sup>*Nkx2.1*<sup>-/-</sup>**i** Cleaved-caspase 3**j** TUNEL

Supplementary figure S4

1249

1250 **Figure 4-figure supplement 1. *Nkx2.I*<sup>-/-</sup> mice brains do not show any increase in**  
1251 **cell death at E16.5.**

1252 **(a-d)** Single immunohistochemical staining for the cleaved-caspase 3 (n=4 for CC  
1253 region and n= 5 for POA region in WT mice; n=6 for CC region and n=10 for POA  
1254 region in *Nkx2.I*<sup>-/-</sup> mice) and **(e-h)** TUNEL staining (n=16 for CC in WT mice, n=22  
1255 for CC in *Nkx2.I*<sup>-/-</sup> mice; n=6 for POA in WT mice, n=5 for POA in *Nkx2.I*<sup>-/-</sup> mice;  
1256 n=10 for MGE in WT mice, n= 11 for MGE in *Nkx2.I*<sup>-/-</sup> mice; n=7 for SEP in WT  
1257 mice, n=14 for SEP in *Nkx2.I*<sup>-/-</sup> mice) on CC coronal sections from wild-type **(a-b**  
1258 **and e-f)** and *Nkx2.I*<sup>-/-</sup> mice **(c-d and g-h)** at E16.5. Cell nuclei were counterstained in  
1259 blue with Hoechst. **b, d, f and h** are higher magnified views of the CC region seen in  
1260 **a, c, e and g**, respectively. **(i and j)** Bars (mean ± SEM from a sample of n=4-16  
1261 sections in the wild-type and n=5-22 sections in *Nkx2.I*<sup>-/-</sup> mice depending on the  
1262 region studied) represent the number of dying cells labelled by the cleaved-caspase 3  
1263 or by the TUNEL staining and displaying pyknotic nuclei per section (surface  
1264 area/section=24119.332 mm<sup>2</sup>), in the CC, POA, MGE and SEP of *Nkx2.I*<sup>-/-</sup> (KO)  
1265 compared to wild-type (WT) mice. No significant differences were observed in the  
1266 number of dying cells in *Nkx2.I*<sup>-/-</sup> mice brains compared to the wildtype. p-value=  
1267 0.1225 for CC and 0.4618 for POA with cleaved caspase 3 staining. p-value= 0.7934  
1268 for CC, 0.8193 for POA, 0.4032 for MGE, and 0.4879 for SEP with TUNEL  
1269 staining. **(CC)** corpus callosum; **(MGE)** medial ganglionic eminence; **(POA)**  
1270 preoptic area; **(SEP)** septum. Bar = 675 μm in **a, c, e and g**; 60 μm in **b and d**; 40  
1271 μm in **f and h**.

1272