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1 **DCC regulates astroglial development essential for telencephalic**
2 **morphogenesis and corpus callosum formation**

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76 **Abstract**

77 The forebrain hemispheres are predominantly separated during embryogenesis by
78 the interhemispheric fissure (IHF). Radial astroglia remodel the IHF to form a
79 continuous substrate between the hemispheres for midline crossing of the corpus
80 callosum (CC) and hippocampal commissure (HC). DCC and NTN1 are molecules
81 that have an evolutionarily conserved function in commissural axon guidance. The
82 CC and HC are absent in *Dcc* and *Ntn1* knockout mice, while other commissures are
83 only partially affected, suggesting an additional aetiology in forebrain commissure
84 formation. Here, we find that these molecules play a critical role in regulating
85 astroglial development and IHF remodelling during CC and HC formation. Human
86 subjects with *DCC* mutations display disrupted IHF remodelling associated with CC
87 and HC malformations. Thus, axon guidance molecules such as DCC and NTN1 first
88 regulate the formation of a midline substrate for dorsal commissures prior to their
89 role in regulating axonal growth and guidance across it.

90

91 **Keywords**

92 Midline zipper glia; astrocyte morphology; agenesis of the corpus callosum; callosal
93 axons; Deleted in colorectal cancer; NTN1, DCC mutations; telencephalic
94 development; interhemispheric fissure remodelling

95

96

97 **Introduction**

98 The corpus callosum (CC) is the largest fibre tract in the human brain and is
99 comprised of approximately 200 million axons (Paul et al., 2007; Tomasch, 1954)
100 connecting similar regions between the left and right cerebral hemispheres (Fenlon
101 and Richards, 2015; Fenlon et al., 2017; Suárez et al., 2018). All eutherian mammals
102 have a CC (Suárez et al., 2014; Suárez, 2017), with malformations or complete
103 absence (agenesis) of the CC occurring in at least 1 in 4000 human live births (Glass
104 et al., 2008). Collectively, these genetically heterogeneous disorders are known as
105 CC dysgenesis, and can result in a wide spectrum of neurological, developmental
106 and cognitive deficits (Brown and Paul, 2019; Edwards et al., 2014; Paul et al.,
107 2007).

108 During brain development, the callosal tract forms between the two
109 telencephalic hemispheres through a midline region initially separated by the
110 interhemispheric fissure (IHF; Gobius et al., 2016; Rakic and Yakovlev, 1968; Silver
111 et al., 1982). Recently, we demonstrated that remodelling of the IHF tissue by
112 specialised astroglial cells, known as midline zipper glia (MZG; Silver et al., 1993), is
113 mediated by FGF8 signalling and subsequent regulation of astroglialogenesis by NFI
114 transcription factors, and is essential to provide a permissive substrate for callosal
115 axons to cross the telencephalic midline (Gobius et al., 2016). MZG are derived from
116 radial glia in the telencephalic hinge, located rostral to the third ventricle. From this
117 ventricular zone, they migrate rostro-dorsally as bipolar cells to the IHF pial surface
118 and transition into multipolar astrocytes. This latter step facilitates their intercalation
119 across the midline and subsequent elimination of the intervening leptomeningeal
120 tissue that comprises the IHF. The MZG thereby fuse the medial septum in a fashion
121 that resembles a 'zipper' mechanism (Gobius et al., 2016), which does not occur in
122 naturally acallosal mammals such as monotremes and marsupials (Gobius et al.,
123 2017). Developmental defects in IHF remodelling invariably result in callosal
124 agenesis in mouse models and, strikingly, all 38 individuals in a human cohort with
125 callosal agenesis also displayed aberrant retention of the IHF and an abnormal
126 separation of the medial septum (Gobius et al., 2016). Thus, the remarkably high
127 prevalence of midline defects in human callosal disorders suggests that there are
128 additional determinant genes for IHF remodelling that have not yet been identified.
129 These could include axon guidance genes, which are frequently mutated in humans
130 (and mice) with CC abnormalities (Edwards et al., 2014).

131 Netrin 1 (NTN1) is a secreted ligand for the deleted in colorectal carcinoma
132 (DCC) receptor, and these molecules function as axon guidance cues in species
133 ranging from *Drosophila* to mammals (Chan et al., 1996; de la Torre et al., 1997;
134 Fazeli et al., 1997; Hedgecock et al., 1990; Keino-Masu et al., 1996; Kolodziej et al.,
135 1996; Serafini et al., 1996). Indeed, NTN1-DCC signalling attracts pioneering callosal
136 axons towards the midline and attenuates chemorepulsive signaling in neocortical
137 callosal axons *ex vivo* to facilitate crossing the midline (Fothergill et al., 2014).
138 Heterozygous and homozygous *DCC* pathogenic variants also result in human
139 callosal dysgenesis at high frequency (Jamuar et al., 2017; Marsh et al., 2018;
140 Marsh et al., 2017) with an estimated incidence of 1 in 14 in unrelated individuals
141 with callosal dysgenesis (Marsh et al., 2017), and *Ntn1* and *Dcc* mouse mutants do
142 not form a CC (Fazeli et al., 1997; Finger et al., 2002; Fothergill et al., 2014; Serafini
143 et al., 1996). Instead of crossing the midline, callosal axons in *Ntn1* and *Dcc* mutant
144 mice form ipsilateral “Probst” bundles that run parallel to the midline (Fazeli et al.,
145 1997; Finger et al., 2002; Fothergill et al., 2014; Ren et al., 2007; Serafini et al.,
146 1996). Together, these results have led to the conclusion that NTN1 and DCC act
147 primarily as axon guidance genes during callosal formation. However, in *Ntn1* and
148 *Dcc* mutant mice, only the CC and hippocampal commissure (HC) are completely
149 absent, while other axon tracts remain intact or are mildly affected (Fazeli et al.,
150 1997; Serafini et al., 1996; Yung et al., 2015), indicating that additional processes
151 might affect the development of the CC and HC in these mice. Moreover, elimination
152 of the leptomeninges, which normally occurs during IHF remodelling (Gobius et al.,
153 2016), is severely disrupted in *Ntn1* mutant mice (Hakanen and Salminen, 2015),
154 further suggesting that NTN1 and its receptor, DCC, may play a hitherto unidentified
155 role in IHF tissue remodelling.

156 Here, we identify a distinct and developmentally earlier role for NTN1 and
157 DCC signalling during CC formation, involving the regulation of MZG development
158 and subsequent IHF remodelling. We find that IHF remodelling is impaired in both
159 *Ntn1* and *Dcc* mouse mutants, as well as in humans with *DCC* pathogenic variants
160 that also display agenesis of the CC and HC. Moreover, in contrast to the wildtype
161 receptor, these human pathogenic variants of *DCC* are unable to regulate cell
162 morphology. Furthermore, we find that defects in astroglial morphology and
163 migration to the IHF in *Ntn1* and *Dcc* mutant mice prevent MZG intercalation and,
164 therefore, IHF remodelling and midline crossing of commissural axons. Taken

165 together, our findings indicate that pathogenic variants in *NTN1* and *DCC* are most
166 likely to affect human CC and HC development through misregulation of astroglial
167 shape, motility and function during IHF remodelling.

168

169 **Results**

170 ***Dcc* signalling is required for IHF remodelling and subsequent CC and HC** 171 **formation**

172 To re-investigate how *Dcc* and *Ntn1* regulate callosal formation, we first analysed the
173 relationship between the IHF and callosal axon growth during midline development in
174 horizontal sections of *Ntn1* and *Dcc* mutant mice. These mouse mutants include *Dcc*
175 knockout, *Dcc^{kanga}* mice, which express a truncated DCC receptor that lacks the P3
176 intracellular signalling domain, and *Ntn1-lacZ* mutant mice, which express reduced
177 levels of NTN1 protein that subsequently becomes sequestered in intracellular
178 organelles (Fazeli et al., 1997; Finger et al., 2002; Fothergill et al., 2014; Serafini et
179 al., 1996). Immunohistochemistry was performed following commissure formation at
180 embryonic day (E)17 against the axonal marker Gap43 together with pan-Laminin,
181 which labels both leptomeningeal fibroblasts and the basement membrane
182 surrounding the IHF (Figure 1A). This revealed that commissural axons in *Dcc*
183 knockout, *Dcc^{kanga}*, and *Ntn1-lacZ* mice remain within the ipsilateral hemisphere and
184 do not form a CC or HC, consistent with previous reports (Fazeli et al., 1997; Finger
185 et al., 2002; Fothergill et al., 2014; Ren et al., 2007; Serafini et al., 1996). We further
186 identified that IHF remodelling had not occurred in *Dcc* knockout, *Dcc^{kanga}*, and *Ntn1-*
187 *lacZ* mice, evidenced by complete retention of the IHF, which separated the majority
188 of the telencephalic midline (Figure 1A). This likely prevented formation of the HC in
189 addition to the CC (Figure 1A). The extent of IHF retention, measured as the ratio of
190 IHF length to total midline length, is significantly larger in *Dcc* and *Ntn1* mutants
191 compared to their wildtype littermates (Supplementary File 1; Figure 1A and 1B), but
192 did not differ between mutants (Supplementary File 1; Figure 1A and 1B). This
193 suggests that NTN1 and DCC may interact or act in a similar manner to regulate IHF
194 remodelling prior to commissural axon crossing, and that the P3 intracellular domain
195 of DCC is crucial for this function. The brain phenotype of adult *Dcc* knockout and
196 *Ntn1-lacZ* mice was unable to be investigated as these animals die shortly after birth
197 (Fazeli et al., 1997; Finger et al., 2002; Serafini et al., 1996). However,
198 immunohistochemistry for the axonal marker Neurofilament in adult *Dcc^{kanga}* mice

199 revealed that the retention of the IHF and absence of the CC and HC persists into
200 adulthood (Supplementary File 1; Figures 1B and Figure 1-figure supplement 1),
201 resembling human congenital callosal agenesis (Edwards et al., 2014; Gobius et al.,
202 2016).

203 We previously reported that humans carrying *DCC* pathogenic variants
204 develop dysgenesis of the CC with incomplete penetrance (Marsh et al., 2017). T1-
205 weighted MRI of four individuals from two unrelated families carrying missense
206 pathogenic variants in *DCC* (p.Val793Gly affecting fibronectin type III-like domain 4
207 of *DCC* and p.Met1217Val; p.Ala1250Thr affecting the cytoplasmic domain of *DCC*;
208 Figure 8A; Marsh et al., 2017), revealed in all cases that the complete absence of
209 the CC was associated with aberrant retention of the IHF and an unfused septum
210 (Figure 1C). Importantly, these individuals were also previously reported to lack a HC
211 (Marsh et al., 2017), suggesting a defect in IHF remodelling may also impact HC
212 development. Since IHF remodelling is required for subsequent callosal axon
213 crossing (Gobius et al., 2016), these results collectively suggest that the underlying
214 cause of callosal agenesis in *Ntn1* and *Dcc* mutant mice and in humans with *DCC*
215 mutations is a failure of IHF remodelling.

216 217 **DCC and NTN1 are expressed by MZG cells throughout interhemispheric** 218 **remodelling**

219 We previously demonstrated that *DCC* is expressed on axons of the CC, HC and the
220 fornix during midline development, while *NTN1* is expressed at the telencephalic
221 midline, within the indusium griseum and the septum but not within callosal axons
222 themselves (Fothergill et al., 2014; Shu et al., 2000). Since our analysis of *Ntn1* and
223 *Dcc* mutant mice revealed that these genes are necessary for IHF remodelling, we
224 then investigated whether they are expressed by the MZG, which mediate IHF
225 remodelling (Gobius et al., 2016). MZG arise in the telencephalic hinge, a region in
226 the septal midline caudal to the IHF and rostral to the third ventricle. Radial glia
227 within the telencephalic hinge are attached to both the third ventricle and the IHF and
228 mature into MZG as they undergo somal translocation to the IHF between E12 and
229 E16 in mice (Gobius et al., 2016). Fluorescent *in situ* hybridization for *Dcc* and *Ntn1*
230 transcripts, combined with immunohistochemistry for the MZG marker *Glast* (Gobius
231 et al., 2016), revealed *Dcc* and *Ntn1* expression in radial MZG progenitor cells within
232 the telencephalic hinge at E12 and E15 (Figure 2B-2D, 2F and 2H; Figure 2-figure

233 supplement 1H-J), and in MZG migrating to the IHF at E15 (Figure 2F and 2H).
234 Furthermore, *Dcc* was expressed in Glast-positive radial glia within the septum but
235 not in the neocortex (Figure 2-figure supplement 1A-C). DCC protein can be
236 identified on Glast-positive processes of radial glia attached to the IHF (Figure 2G),
237 which are adjacent to Gap43-positive axons traversing the midline region that also
238 express DCC (Figure 2-figure supplement 1E). Following IHF remodelling at E17,
239 mature Gfap-positive/Sox9-positive multipolar MZG cells (Gobius et al., 2016; Sun et
240 al., 2017) and Glast-positive MZG cells within the telencephalic hinge continue to
241 express DCC (Figure 2J-2L). A comparison of DCC immunohistochemistry in
242 wildtype and *Dcc* knockout mice confirmed that the antibody specifically recognised
243 DCC protein within both commissural axons and MZG cells (Figure 2-figure
244 supplement 1K). Importantly, we did not observe specific staining for either *Dcc* or
245 *Ntn1* mRNA within the IHF (including the leptomeninges) at any stage analysed
246 (Figures 2 and S2).

247 Since NTN1 is a secreted cue (Kennedy et al., 1994; Sun et al., 2011), we
248 investigated which cells express NTN1, and where secreted NTN1 may be
249 deposited, by comparing patterns of immunohistochemistry for β -galactosidase (β -
250 gal) and NTN1 antibodies in heterozygous and homozygous *Ntn1-lacZ* mutants, in
251 which NTN1 is fused to a β -gal and trapped in intracellular compartments (Serafini et
252 al., 1996). NTN1/ β gal-positive puncta were enriched in Glast-positive MZG cells in
253 *Ntn1-lacZ* mice (Figure 2I). Furthermore, we identified NTN1 protein on the IHF
254 basement membrane (Figures 2I and S2G), on growing commissural axons (Figure
255 2-figure supplement 1G), and on MZG membranes in control heterozygotes, but not
256 in *Ntn1-lacZ* homozygous mutant mice (Figure 2I). Therefore, MZG cells produce
257 and secrete NTN1 that becomes deposited on the basement membrane of the IHF,
258 on commissural axons, and on MZG cell processes in the region of initial IHF
259 remodelling (Figure 2E). Collectively, our results demonstrate that both *Ntn1* and
260 *Dcc* are expressed by MZG, and suggest that autocrine NTN1-DCC signalling may
261 regulate MZG development and subsequent IHF remodelling.

262

263 ***Dcc* signalling regulates MZG cell morphology and process organisation prior**
264 **to IHF remodelling**

265 Two key steps in IHF remodelling are the somal translocation of radial MZG
266 progenitors to the IHF, and their subsequent transition into multipolar MZG cells that
267 intercalate across the midline (Gobius et al., 2016). As both NTN1 and DCC are
268 expressed by MZG, we next asked whether these molecules regulate MZG
269 development. Immunohistochemistry for Nestin and Glast, which are markers of
270 radial MZG, revealed distinct differences in MZG development in *Dcc^{kanga}* mice from
271 E14 onward, but not in radial MZG progenitors at E13 (Figure 3-4 A). In wildtype
272 mice, the endfeet and cell bodies of radial Glast-positive MZG cells are evenly
273 distributed along the medial septum and adjacent to the pial surface of the IHF
274 (Figures 3B, 3D, 4A and 4C). However, in *Dcc^{kanga}* mutants, radial MZG accumulate
275 at the base of the IHF (Figure 3A-D). Furthermore, long radial Nestin-positive MZG
276 processes extending from the ventricular zone to the rostral-most pial surface of the
277 IHF are noticeably absent from *Dcc^{kanga}* mutants, and instead, Nestin-positive
278 *Dcc^{kanga}* processes cluster close to the rostral IHF pial surface and appear
279 disorganised (Figures 3A, 3C, 3C', 3E and 3-figure supplement 1B-D). These
280 abnormalities were further quantified as a significant increase in fluorescence
281 intensity of Glast staining within the base of the IHF, and a concomitant decrease in
282 the region 150-200 μm distant from the IHF base in *Dcc^{kanga}* mutants, compared to
283 their wildtype littermates at E14 (Supplementary File 1; Figure 3B, 3B' and 3G). Just
284 prior to IHF remodelling at E15, there was an overall decrease in Glast-positive
285 radial MZG processes in *Dcc^{kanga}* mutants (Figure 3C, 3H and Supplementary File 1).
286 While there was no difference in fluorescence intensity of Glast-positive radial MZG
287 processes one day later at E16, *Dcc^{kanga}* MZG processes continued to display
288 irregular morphology and failed to intercalate across the IHF (Figure 3E, 3F, 3I and
289 Supplementary File 1). Interestingly, we identified a similar defect in the distribution
290 of Glast-positive MZG processes in *Ntn1-lacZ* mutant mice at E15 (Figure 3K).
291 These results suggest that both DCC and NTN1 are required for the correct
292 morphology and distribution of MZG processes prior to IHF remodelling. Moreover,
293 abnormal morphology and increased abundance of GLAST-positive and NESTIN-
294 positive radial fibers of the dorsal glial population, known as the glial wedge, was
295 also evident in E15 *Dcc^{kanga}* mice (Figure 3C-D), suggesting DCC regulates the
296 morphology and distribution of at least 2 midline glial populations prior to CC
297 development.

298 To further characterise the defect in MZG cell distribution in *Dcc^{kanga}* mice, we
299 then measured the maximum rostro-caudal extent to which MZG occupy the IHF pial
300 surface, and normalised this value to the total midline length from E14-E16 (Figure
301 3A-F and 3J). The distribution of Nestin-positive and Glast-positive MZG along the
302 IHF was significantly decreased at both E14 and E15 in *Dcc^{kanga}* mice compared to
303 their wildtype littermates (Figure 3A-D, 3J and Supplementary File 1). The
304 attachment of MZG processes to the IHF pial surface is therefore specifically
305 reduced in the rostral region of the IHF prior to IHF remodelling in *Dcc^{kanga}* mice. This
306 may impact the directed somal translocation of *Dcc^{kanga}* MZG cell bodies and their
307 subsequent distribution along the IHF surface prior to IHF remodelling.

308 Next, we further investigated whether the aberrant organisation of radial glial
309 processes along the IHF in *Dcc^{kanga}* mice was due to a loss of endfoot adhesion to
310 the IHF pial surface. There was no difference in fluorescence intensity of Nestin-
311 positive MZG processes within 5 μm adjacent to the IHF surface between *Dcc^{kanga}*
312 and wildtype mice, suggesting comparable attachment of radial glial endfeet to the
313 IHF in both strains (Supplementary File 1, Figure 3-figure supplement 1E and G).
314 This was further evidenced by the normal localisation of α - and β -dystroglycan at the
315 pial IHF surface in *Dcc^{kanga}* mice, where these molecules form crucial adhesions
316 between radial glial endfeet and the extracellular matrix (Myshrall et al., 2012;
317 Supplementary File 1; Figure 3-figure supplement 1-D). Moreover, molecules that
318 normally maintain the bipolar morphology of radial glia, such as β -catenin and N-
319 cadherin, were also expressed normally within *Dcc^{kanga}* Nestin-positive radial glia,
320 but adenomatous polyposis coli (APC) was instead significantly reduced in *Dcc^{kanga}*
321 Nestin-positive radial glial endfeet (Supplementary File 1, Figure 3-figure supplement
322 1D and 3-figure supplement 1E; Yokota et al., 2009). APC regulates the growth and
323 extension of basal radial glial processes and cell polarity of radial glia and migrating
324 astrocytes (Etienne-Manneville and Hall, 2003; Yokota et al., 2009). Thus, reduced
325 localisation of APC within *Dcc^{kanga}* radial glial basal endfeet may indicate perturbed
326 regulation of cell process growth and/or cell polarity. Therefore, *Dcc^{kanga}* Nestin-
327 positive radial glia display reduced elongation and reduced occupation of the pial IHF
328 surface compared to wildtype radial progenitors of MZG. Collectively, these results
329 suggest that DCC is not required for radial MZG to adhere to the IHF, but instead
330 regulates the morphology and organisation of radial MZG processes along the pial
331 IHF surface prior to IHF remodelling.

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Dcc signalling regulates MZG somal translocation to the IHF prior to IHF remodelling

To determine if the aberrant morphology and organisation of radial glial processes observed in *Dcc^{kanga}* mice affects the subsequent distribution of translocated MZG cell bodies at the IHF surface, immunohistochemistry for glial markers Sox9 and Glast was performed in E14-E16 *Dcc^{kanga}* mice. Wildtype MZG undergo substantial somal translocation to the IHF between E14 and E15 (Gobius et al., 2016; Supplementary File 1, Figure 4A, 4C and 4G). In contrast, *Dcc^{kanga}* mice showed reduced somal translocation to the IHF (Supplementary File 1; Figure 4A, 4C and 4G), with significantly fewer MZG cells at the IHF pial surface by E15 in *Dcc^{kanga}* compared to wildtype mice (Supplementary File 1; Figure 4B and 4G). When binned along the rostro-caudal axis, we found a significant reduction in the number of cell bodies reaching the rostral IHF pial surface in E15 *Dcc^{kanga}* mice (200-250 μ m; Supplementary File 1 and Figure 4C-D). Since MZG progenitors somal translocate toward their basal process attached to the IHF (Gobius et al., 2016), our results suggest that the lack of radial MZG processes occupying the rostral E14 IHF surface in *Dcc^{kanga}* mice results in a decrease of MZG cell bodies present in the corresponding region one day later. There was, however, a significant increase in MZG cell bodies present at the IHF pial surface between E15 and E16 in *Dcc^{kanga}* mice (Supplementary File 1, Figure 4C, 4E and 4G). This suggests that MZG migration towards the IHF is delayed but does eventually occur in *Dcc^{kanga}* mice, albeit after IHF remodelling would normally have been initiated. *Dcc^{kanga}* MZG remain adjacent to the unremodelled IHF at E16 in contrast to wildtype MZG, which are scattered along the midline where IHF remodelling has occurred and continue to expand their domain rostral and dorsal for further IHF remodelling (Gobius et al., 2016). Furthermore, despite DCC having been previously implicated in regulating cell proliferation and cell death (Arakawa, 2004; Llambi et al., 2001; Mehlen et al., 1998), cell birth-dating, differentiation and apoptosis experiments did not reveal any significant differences between the MZG of *Dcc^{kanga}* and wildtype mice (Supplementary File 1 and Figure 4-figure supplement 1). Taken together, these results suggest that the irregular morphology and distribution of radial *Dcc^{kanga}* MZG processes is associated with delayed somal translocation of MZG to the IHF surface, and may prevent the initiation of IHF remodelling.

366 Radial glia in the corticoseptal boundary detach from the pial surface and
367 cluster their processes to form a triangular group of cells known as the glial wedge,
368 while other radial glia in this region translocate their soma to the IHF (similar to MZG
369 cells), where they subsequently form the indusium griseum glia (Shu and Richards,
370 2001; Smith et al., 2006). We investigated whether DCC also regulates the
371 development of these glial populations, which were observed to be abnormal at E15
372 (Figure 3C-D) and secrete axon guidance molecules during CC formation (reviewed
373 in Donahoo and Richards, 2009; Gobius and Richards, 2011; Morcom et al., 2016).
374 In *Dcc^{kanga}* and *Dcc* knockout mice, the glial wedge was malformed and there was a
375 major reduction in somal translocation of Sox9-positive indusium griseum glia to the
376 IHF surface, which subsequently prevented formation of this glial guidepost cell
377 population (Supplementary File 1 and Figure 4-figure supplement 1G-I). Thus, DCC
378 may play a more general role in regulating the morphological maturation and
379 migration of multiple radial astroglial populations in the developing midline, which are
380 critical for CC formation.

381

382 **DCC signalling regulates MZG cell morphology and spatial distribution during** 383 **IHF remodelling**

384 We previously demonstrated that MZG differentiation is controlled by molecular
385 signaling initiated by the morphogen FGF8 via the Mitogen activated protein kinase
386 (MAPK) pathway to NFI transcription factors A and B (Gobius et al., 2016). Members
387 of this signaling pathway (*Fgf8*, NFIA, NFIB, and p-ERK1/2) were expressed
388 normally in *Dcc^{kanga}* MZG compared to MZG in their wildtype littermates at E15
389 (Figure 5-figure supplement 1B, S5D-F and Supplementary File 1). Further, *Dcc^{kanga}*
390 MZG continue to express *Mmp2* mRNA (Figure 5-figure supplement 1C, D and
391 Supplementary File 1), which we previously demonstrated to be expressed during
392 MZG-mediated degradation of the IHF during remodelling (Gobius et al., 2016).

393 Next, we investigated the distribution and maturation of MZG in *Ntn1* and *Dcc*
394 mutant mice at E16 and 17, when wildtype MZG normally differentiate into multipolar
395 astrocytes during IHF remodelling (Gobius et al., 2016). Immunohistochemistry for
396 Nestin, Glast (Figures 3F, 3J) and Gfap (Figure 5A) demonstrated that *Dcc^{kanga}*, *Dcc*
397 knockout and *Ntn1-lacZ* MZG remain attached to the caudal IHF pial surface and
398 have not intercalated at stages equivalent to when wildtype MZG have infiltrated and
399 remodelled the IHF (Figures 3F, 3J, 5A and Supplementary File 1). DCC-deficient

400 MZG expressed GFAP at comparable levels to wildtype MZG at E17, and
401 demonstrated no precocious expression of GFAP at E15, similar to wildtype MZG
402 (Figure 5A-B, Figure 5-figure supplement 1A and Supplementary File 1). Therefore,
403 DCC-deficient MZG do not mature precociously prior to migration and IHF
404 remodelling, or fail to differentiate during callosal development. However, *Ntn1-lacZ*,
405 *Dcc^{kanga}* and *Dcc* knockout mice demonstrate a significant reduction of GFAP-
406 positive glia at E17 in the region where the CC normally forms in wildtype mice (i.e.,
407 > 450 μm from the third ventricle; Figure 5A-C and Supplementary File 1). Instead,
408 MZG in these mutants remain close to the third ventricle and the majority fail to
409 migrate. To quantify this, we normalised the level of GFAP between sections and
410 calculated a rostro-caudal ratio of this fluorescence. We observed a significant
411 reduction in the rostro-caudal ratio of GFAP fluorescence in *Ntn1-lacZ*, *Dcc^{kanga}* and
412 *Dcc* knockout mice compared to controls (Figure 5A-D and Supplementary File 1).
413 Since progressive migration and intercalation of MZG is required for IHF remodelling
414 (Gobius et al., 2016), these results indicate that *Ntn1* and *Dcc* affect IHF remodelling
415 by regulating the morphology and spatial organisation of both radial MZG progenitors
416 and mature MZG, and therefore their ability to intercalate across the IHF, but not
417 their proliferation and adhesion to the pial IHF surface.

418

419 **Variable DCC knockdown during midline development causes a spectrum of** 420 **callosal phenotypes.**

421 The current and previous results from our laboratory indicate at least two distinct
422 roles for NTN1 and DCC during CC formation: first, they act on astroglia to facilitate
423 remodelling of the IHF, and second, they regulate the pathfinding of callosal axons to
424 the telencephalic midline (Fothergill et al., 2014). To investigate these roles further,
425 we aimed to disrupt DCC expression specifically within the progenitors of callosal
426 neurons, sparing expression within MZG. We designed two *Dcc*-targeted
427 CRISPR/CAS9 constructs (*Dcc*-CRISPR) and acquired a *Dcc*-targeted shRNA (*Dcc*-
428 shRNA; Zhang et al., 2018) for targeted in utero electroporation into the E13
429 cingulate cortex in wildtype and *Dcc^{kanga}* mice and successfully labelled callosal
430 axons that reach the contralateral hemisphere by E18 (Figure 6-figure supplement
431 1A). We observed no phenotype in all experimental cases (Figure 6-figure
432 supplement 1A), and instead found these techniques failed to reduce DCC
433 expression sufficiently; the only significant reduction in DCC protein was observed in

434 heterozygous *Dcc*^{kanga} mice electroporated with *Dcc*-shRNA (average DCC
435 expression reduced to 93.06% compared to the non-electroporated hemisphere;
436 Figure 6-figure supplement 1A-C and Supplementary File 1). In order to knockout
437 DCC more robustly in the cortex, we crossed *Dcc*^{flox/flox} mice (Krimpenfort et al.,
438 2012) with mice carrying *Emx1*^{iCre} (Kessar et al., 2006), and the *tdTomato*^{flox-stop}
439 reporter allele (Madisen et al., 2010).

440 At birth, we observed a spectrum of callosal phenotypes in *Dcc* cKO mice,
441 including complete callosal absence (4/12 mice), partial CC absence (5/12 mice),
442 and a normal CC that was comparable to control mice, which do not express
443 *Emx1*^{iCre} (3/12 mice) based on rostral-caudal CC length across ventral, middle and
444 dorsal horizontal sections (Figure 6A and 6F). Moreover, the HC was significantly
445 reduced in the majority of animals and was absent in one *Dcc* cKO mouse (Figure
446 6A, 6H and Supplementary File 1). Unexpectedly, we found the IHF was significantly
447 retained across *Dcc* cKO mice, indicating that IHF remodelling had not been
448 completed (Figure 6A, 6D-E, and Supplementary File 1). The severity of callosal
449 agenesis and HC dysgenesis was significantly correlated with the extent to which the
450 IHF had been remodelled (Figure 6A, 6J, 6K, Figure 6-figure supplement 1D-G and
451 Supplementary File 1). Complete callosal agenesis *Dcc* cKO mice demonstrated the
452 most severe retention of the IHF, encompassing the majority of the telencephalic
453 midline, while partial callosal agenesis and even full CC *Dcc* cKO mice demonstrated
454 a retention of the rostral IHF (Figure 6A, 6E and Supplementary File 1). Moreover, in
455 partial callosal agenesis and full CC *Dcc* cKO mice that demonstrated partial
456 retention of the rostral IHF, callosal axons often crossed the midline more caudal in a
457 region where the IHF had been remodelled compared to control mice (see corpus
458 callosum remnant or CCR in Figure 6A). This suggests that in the absence of their
459 normal substrate, callosal axons are able to adapt and cross the midline in a region
460 where the substrate is available. These results were reflected by a significant
461 increase in the rostro-caudal depth of the partial or full CC in *Dcc* cKO mice (Figure
462 6A, 6G and Supplementary File 1). In *Dcc* cKO mice with complete callosal
463 agenesis, callosal axons were unable to cross the midline and accumulated adjacent
464 to the IHF that had not been remodelled (red arrowheads in Figure 6A), similar to
465 *Dcc*^{kanga} and *Dcc* knockout mice. These results demonstrate that DCC regulates the
466 extent of IHF remodelling throughout the telencephalic midline. The retention of the
467 IHF in these mice was unexpected; we had instead expected that reduced DCC

468 expression in the cortex would cause callosal axon misguidance with normal
469 formation of an interhemispheric substrate. Instead, our results suggest that DCC
470 primarily regulates the formation of the interhemispheric substrate to determine CC
471 size in mice.

472 We next explored whether loss of DCC expression within callosal axons might
473 cause prior callosal axon misguidance that could indirectly impact IHF remodelling.
474 We found that DCC expression was significantly reduced in the cingulate cortex and
475 adjacent intermediate zone in the majority of P0 *Dcc* cKO mice (mean expression
476 reduced to 80.4% and 62.9% respectively; Figure 6A, 6I and Supplementary File 1),
477 and within E15 *Dcc* cKO mice (mean DCC expression in the cingulate cortex
478 reduced to 76.6% in *Dcc* cKO; Figure 6L, 6O and Supplementary File 1), as
479 expected. Surprisingly, we found that TDTOMATO-positive/GAP43-positive axons,
480 which had reduced DCC expression, approached the interhemispheric midline in *Dcc*
481 cKO mice, similar to their cre-negative littermates (Figure 6M-N, 6P and
482 Supplementary File 1). This suggests that axons with reduced, but not entirely
483 eliminated DCC expression, approach the midline adjacent to the IHF in a timely and
484 spatially appropriate manner, and are unable to cross the midline in regions where
485 the IHF is not remodelled in *Dcc* cKO mice.

486 Next, we investigated the recombination pattern and development of MZG in
487 *Dcc* cKO mice. Cre activity, as measured by TDTOMATO expression, was
488 widespread in cells throughout the telencephalic midline, including septal cells and
489 HC axons, resulting in reduced DCC expression in multiple cell types (Figure 6B).
490 Mean DCC expression within the telencephalic hinge was comparable between *Dcc*
491 cKO mice and their cre littermates (Figure 6L, 6O and Supplementary File 1), but we
492 also observed TDTOMATO-positive/GLAST-positive MZG cell bodies within the
493 telencephalic hinge, and at the IHF surface in *Dcc* cKO mice (Figure 6Q-R and
494 Supplementary File 1). This suggests the potential for *Dcc* knockdown in a subset of
495 MZG cells within *Dcc* cKO mice, which may fail to intercalate across the IHF,
496 possibly causing the IHF remodelling defect observed in P0 *Dcc* cKO mice.
497 However, unlike *Dcc*^{kanga} mice, we were unable to find a significant population
498 difference in the distribution of GLAST-positive MZG between *Dcc* cKO mice and
499 their cre-negative littermates, at the level of DCC knockdown observed in this model
500 (Figure 6-figure supplement 1H-I and Supplementary File 1). Thus, the variable
501 callosal and IHF remodelling phenotypes observed in *Dcc* cKO mice likely arise from

502 varying degrees of DCC knockdown in these models due to the mosaic expression
503 of *Emx1*^{iCre} within MZG. In order to further explore the role of DCC and the impact of
504 human *DCC* mutations on the behavior of astroglia, we next investigated the function
505 of human *DCC* mutations using in vitro assays.

506
507 **NTN1-DCC signalling promotes cytoskeletal remodelling of astroglia and**
508 **neural progenitors**

509 Our results suggest that NTN1 and DCC may have important functions in the
510 morphological development of radial glia more broadly. We established *in vitro*
511 assays to test the function of NTN1-DCC signalling and DCC mutant receptors in
512 regulating the morphology of astroglial-like cells. Such assays can also be used to
513 examine human variants of DCC pathogenic mutations (see next section). To
514 develop these assays, we employed N2A neuroblast cells, which display neural
515 progenitor properties (Augusti-Tocco and Sato., 1969; Shea et al., 1985), as well as
516 U251 glioma cells, which express astroglial markers and display invasive capacity
517 (Zhang et al., 2013) similar to MZG cells. Importantly, endogenous DCC has
518 previously been demonstrated to render several glioma cell lines migratory in
519 response to a gradient of NTN1 as a chemoattractant (Jarjour et al., 2011). Both cell
520 lines were transfected with either full-length DCC fused to a TDTOMATO reporter
521 (pCAG-DCC:TDTOMATO) to express wildtype DCC, or a membrane-targeted
522 TDTOMATO reporter (pCAG-H2B-GFP-2A-Myr-TDTOMATO) as a control and
523 stimulated with NTN1. Moreover, we transfected U251 cells with a DCC^{kanga}
524 construct (pCAG-DCC^{kanga}:TDTOMATO), to test whether the P3 domain was critical
525 for NTN1-DCC signalling effects on cell morphology.

526 Expression of DCC:TDTOMATO in U251 cells in the absence of ligand
527 (vehicle alone) promoted cell spreading and elongation, reflected by a significant
528 increase in average cell area and cell perimeter, and a significant decrease in cell
529 circularity compared to control (Supplementary File 1 and Figure 7A-F). This effect
530 was not observed following expression of the DCC^{kanga} construct alone
531 (Supplementary File 1 and Figure 7A-F), suggesting that the P3 domain of DCC is
532 critical for inducing changes in glial cell shape. We further confirmed that these
533 morphological changes were due to the presence of the coding region of wildtype
534 DCC, by comparing to cells transfected with plasmids where DCC had been excised
535 and only the TDTOMATO remained (pCAG-TDTOMATO; Supplementary File 1 and

536 Figure 7-figure supplement 1A and Figure 6-figure supplement 17B). A similar effect
537 was observed following DCC overexpression in N2A cells, which also registered a
538 significant increase in average cell area and cell perimeter, and decrease in cell
539 circularity, compared to controls (Supplementary File 1, Figure 7G-I, and Figure 7-
540 figure supplement 1G and H), further indicating similar effects on cell morphology in
541 glial and neural progenitor lineages. Interestingly, application of NTN1 did not affect
542 cell shape following DCC expression in either cell line (Supplementary File 1 and
543 Figure 7A-F), suggesting that endogenous NTN1, which is known to be expressed
544 by U251 cells (Chen et al., 2017), may be sufficient for activation of
545 DCC:TDTOMATO receptors, or that NTN1 is not required for this effect. To
546 investigate this, we examined NTN1 expression in these cell lines using western blot
547 analysis. We confirmed that both our cell lines expressed NTN1 endogenously, and
548 that transfection of DCC increased DCC levels but did not affect NTN1 expression
549 (Figure 7-figure supplement 1J). No endogenous DCC was detected by western blot
550 in either cell line (Figure 7-figure supplement 1J). Thus, addition of DCC induced
551 cytoskeletal rearrangements in both N2A and U251 cells, which may involve
552 autocrine NTN1 signalling. Typical features of DCC-expressing cells with or without
553 bath application of NTN1 included actin-rich regions resembling filopodia,
554 lamellipodia, and membrane ruffling in U251 cells, while only filopodia were highly
555 abundant in DCC:TDTOMATO-expressing N2A cells; all of these features were
556 rarely observed in control cells from both cell lines (Figure 7A, G). No difference in
557 cleaved-caspase 3-mediated cell apoptosis was observed following DCC expression
558 in either cell line (Figure 7-figure supplement 1I). This suggests that DCC signalling
559 does not mediate programmed cell death but rather promotes remodelling of the
560 actin cytoskeleton in glioma and neuroblast cells in a similar manner to neurons and
561 oligodendrocytes (Rajasekharan et al., 2009; Shekarabi and Kennedy, 2002).

562

563 **Humans with agenesis of the CC carry loss-of-function pathogenic variants in** 564 ***DCC* that are unable to modulate cell shape**

565 Having established that DCC signalling rearranges the cytoskeleton of astroglial-like
566 cells, and that the P3 domain of DCC is crucial for this function, we next investigated
567 whether *DCC* mutant receptors from humans with dysgenesis of the CC affected this
568 function. Site directed mutagenesis was performed to introduce missense mutations
569 into the pCAG-DCC:TDTOMATO expression vector in order to model mutated *DCC*

570 receptors found in six families with previously reported cases of complete or partial
571 agenesis of the CC (p.Met743Leu, p.Val754Met, p.Ala893Thr, p.Val793Gly,
572 p.Gly805Glu, p.Met1217Val;p.Ala1250Thr; Marsh et al., 2017; Marsh et al., 2018;
573 Figure 8A and Figure 6-figure supplement 1C). We further included two artificial
574 mutant receptors that were previously shown to perturb NTN1 binding and
575 chemoattraction (p.Val848Arg, p.His857Ala; Finci et al., 2014). First, these mutants
576 were transfected into HEK293T and COS-7 cells that do not endogenously express
577 DCC (Chen et al., 2013; Shekarabi and Kennedy, 2002). Immunoblotting and
578 immunohistochemistry performed without cell permeabilisation revealed that all
579 mutant DCC receptors were appropriately expressed and localised to the cell
580 membrane (Gad et al., 2000; Figure 7-figure supplement 1E). Using a previously
581 established *in vitro* binding assay (Müller and Soares, 2006; Zelina et al., 2014), we
582 discovered that DCC mutant proteins with altered residues located at the NTN1
583 binding interface (p.V793G and p.G805E) were unable to bind NTN1 (Figure 8B),
584 while all other receptors with altered residues lying outside of the NTN1 binding
585 interface still bound NTN1 (p.M743L, p.V754M, p.A893T and p.M1217;A1250T;
586 Figure 8B). Surprisingly, all eight mutant *DCC* receptors were unable to modulate
587 cell morphology in the presence of NTN1 (Figure 8C-E; Supplementary File 1).
588 Collectively, our results suggest a model whereby mutations that affect the ability for
589 DCC to regulate cell shape (Figure 8F), are likely to cause callosal agenesis through
590 perturbed MZG migration and IHF remodelling.

591

592 **Discussion**

593 Genes that encode axon guidance molecules frequently cause callosal dysgenesis
594 when knocked out in mice (Edwards et al., 2014). This has led to the prevalent view
595 that callosal dysgenesis in these mice might be primarily to due defects in callosal
596 axon guidance towards and across the midline. Here, we identified a novel function
597 for the classical axon guidance genes NTN1 and DCC in regulating the morphology
598 of midline astroglia for IHF remodelling prior to CC and HC formation. Importantly,
599 normal astroglial development and IHF remodelling are critical processes that
600 precede and are necessary for subsequent CC axon guidance across the
601 interhemispheric midline (Gobius et al., 2016). We find that defects in IHF
602 remodelling are consistently associated with dysgenesis of the CC and HC in mice
603 and humans with pathogenic variants in *Ntn1* or *Dcc*.

604 Our *in vitro* assays and analysis of mouse and human cell morphology
605 indicate that the cytoskeletal remodelling function of NTN1-DCC signalling is likely to
606 be crucial for MZG development, IHF remodelling, and subsequent CC formation.
607 The timely differentiation and appropriate distribution of MZG cells at the IHF surface
608 is required for their intercalation and IHF remodelling function (Gobius et al., 2016).
609 Our data suggest a model whereby failed IHF remodelling associated with mutations
610 in *Ntn1* and *Dcc* occurs due to delayed astroglial migration to the IHF as a
611 consequence of perturbed process extension and organisation of MZG precursors.
612 Notably, no medial extension of MZG processes across the basement membrane or
613 perforations in the IHF to allow glia from each hemisphere to interact and intercalate
614 were observed in *Ntn1* or *Dcc* mutant mice at any developmental stage examined.
615 This suggests that NTN1-DCC signalling might also be required for MZG
616 intercalation and removal of the intervening leptomeninges. The DCC homologue
617 UNC-40 is known to facilitate formation of a polarised actin-rich cell protrusion in the
618 *Caenorhabditis elegans* anchor cell, which breaches the basement membrane rich in
619 UNC-6 (NTN1 homolog), enabling the cell to invade the vulval epithelium (Hagedorn
620 et al., 2013; Ziel et al., 2009). DCC may perform a similar function in MZG by
621 engaging secreted NTN1, which we found to be localised at the IHF basement
622 membrane in agreement with the localisation of radial-glia-derived NTN1 in the
623 spinal cord (Varadarjan et al., 2017), and preferentially polarising actin remodelling
624 and process extension toward the IHF during MZG intercalation. Moreover, callosal
625 axons that also rely on DCC-mediated cytoskeletal remodelling for growth and
626 guidance, may non-cell-autonomously influence the final stages of MZG
627 development via a secreted cue or contact-dependent mechanism. Further
628 dissecting this would ideally involve even greater precision in complete and cell-type
629 specific knockout of DCC and NTN1, since knockdown in a subset of cells or merely
630 lowering the expression level was insufficient to induce a consistent phenotype.

631 Notably, we find that the P3 domain-dependent functions of DCC may be
632 required for astroglial development and IHF remodelling. These functions include
633 receptor dimerisation, interaction with the co-receptor ROBO1, or interaction with
634 effectors FAK, MYO10, and TUBB3 (Fothergill et al., 2014; Li et al., 2004; Qu et al.,
635 2013; Stein and Tessier-Lavigne, 2001; Wei et al., 2011; Xu et al., 2018).
636 Accordingly, mice deficient in *Robo1*, *Fak* and *Tubb3*, as well as their signaling
637 effectors *Cdc42*, *Fyn*, *Enah* and *Mena*, which normally act downstream of DCC to

638 regulate the cell cytoskeleton, all display dysgenesis of the CC (Andrews et al.,
639 2006; Beggs et al., 2003; Goto et al., 2008; Menzies et al., 2004; Tischfield et al.,
640 2010; Yokota et al., 2010). Similarly, astroglial cells remodel their cytoskeleton to
641 transition from a bipolar to multipolar morphology, and this process is known to
642 involve the intracellular DCC effectors CDC42, RAC1, RHOA, N-WASP and EZRIN
643 (Abe and Misawa, 2003; Antoine-Bertrand et al., 2011; Derouiche and Frotscher,
644 2001; Lavielle et al., 2011; Murk et al., 2013; Racchetti et al., 2012; Shekarabi et al.,
645 2005; Zeug et al., 2018). Whether these molecules serve as downstream effectors of
646 DCC to influence astroglial development and IHF remodelling during CC formation is
647 an interesting question for future research.

648 In addition to NTN1 and DCC, as shown here, mice lacking the axon guidance
649 molecules ENAH, SLIT2, SLIT3, and RTN4R have previously been reported to have
650 incomplete IHF remodelling and disrupted midline glial development associated with
651 callosal dysgenesis (Menzies et al., 2004; Unni et al., 2012; Yoo et al., 2017). Taken
652 together, those studies and ours suggest that other axon guidance genes may play
653 similar roles in astroglial development and IHF remodelling during CC formation.
654 Additional candidate axon guidance molecules that may regulate IHF remodelling
655 include EPHB1, EFNB3, GAP43, HS6ST1, HS2ST1, ROBO1 and VASP, since
656 mouse mutants lacking these molecules display disrupted midline glial development
657 and callosal dysgenesis (Andrews et al., 2006; Conway et al., 2011; Mendes et al.,
658 2006; Menzies et al., 2004; Shen et al., 2004; Unni et al., 2012). Additional
659 molecules of interest are EFNB1, EFNB3, EPHB2, and EPHA4, since these are
660 expressed by MZG (Mendes et al., 2006).

661 In summary we have demonstrated that rather than solely regulating axon
662 guidance during telencephalic commissure formation, *Dcc* and *Ntn1* are critical
663 genes required for IHF remodelling. Moreover, our study provides a novel role for
664 axon guidance receptor DCC in regulating astroglial morphology, organisation and
665 migration. Exemplified by *Ntn1* and *Dcc*, our study provides support for widespread
666 consideration of astroglial development and IHF remodelling as possible underlying
667 mechanisms regulated by these and other classically regarded “axon guidance
668 genes” during CC formation.

669

670 **Materials and Methods**

671 **EXPERIMENTAL MODELS AND SUBJECT DETAILS**

672 *Animals*

673 *Dcc*^{flox/flox} (Krimpenfort et al., 2012), *Dcc* knockout (Fazeli et al., 1997), *Dcc*^{kanga}
674 (Finger et al., 2002), *Emx1*^{iCre} (Kessarlis et al., 2006), *Ntn1-lacZ* (Serafini et al., 1996,
675 and *tdTomato*^{flox-stop} (Madisen et al., 2010) mice on the C57BL/6J background and
676 CD1 wildtype mice were bred at The University of Queensland. Prior approval for all
677 breeding and experiments were obtained from the University of Queensland Animal
678 Ethics Committee. Male and female mice were placed together overnight and the
679 following morning was designated as E0 if a vaginal plug was detected. *Dcc*
680 knockout and *Dcc*^{kanga} mice were genotyped by PCR and *Ntn1-lacZ* mice were
681 tested for the presence of the *LacZ* gene and deemed homozygous if the β -
682 galactosidase enzyme was trapped intracellularly, as previously described (Fazeli et
683 al., 1997; Finger et al., 2002; Fothergill et al., 2014; Krimpenfort et al., 2012; Serafini
684 et al., 1996). *Dcc*^{flox/flox} mice were genotyped by the Australian Equine Genetics
685 Research Centre at the University of Queensland.

686

687 *Human subjects*

688 Ethics for human experimentation was acquired by local ethics committees at The
689 University of Queensland (Australia), the Royal Children's hospital (Australia), and
690 UCSF Benioff Children's Hospital (USA). Genetic studies were performed previously
691 (Marsh et al., 2017). Structural MR images were acquired as previously described
692 (Marsh et al., 2017). In our study, we analysed the brain phenotype of affected
693 individuals in family 2 (carrying *DCC* p.Val793Gly) and family 9 (carrying *DCC*
694 p.Met1217Val;p.Ala1250Thr in cis) from our previous study.

695

696 **METHOD DETAILS**

697 *Cell birth-dating and tissue collection*

698 For cell birth dating studies, 5-ethynyl-2'-deoxyuridine (EdU; 5 mg per kg body
699 weight, Invitrogen) dissolved in sterile phosphate buffer solution (PBS) was injected
700 into the intraperitoneal cavity of awake pregnant dams. Brains were fixed via
701 transcardial perfusion or immersion fixation with 4% paraformaldehyde (PFA).

702

703 *Cell lines*

704 HEK293 cells (from ATCC CRL-1573, not authenticated, free of mycoplasma
705 contamination) were used to express alkaline phosphatase-conjugated NTN1

706 (NTN1-AP) in the supernatant of COS-7 cell culture. Although this cell line is
707 commonly misidentified, this did not affect the conclusion of the binding assay done
708 in COS-7 cells. U251 cells were obtained as U-373MG (RRID: CVCL_2219) but
709 subsequently identified as U-251 via PCR-based short tandem repeat profiling. All
710 cell lines were routinely tested for mycoplasma to ensure that cell lines were free of
711 mycoplasma contamination. See the key resources table (supplementary file 2) for
712 more information.

713

714 *Cell culture*

715 All cell lines were cultured at 37°C within a humidified atmosphere containing 5%
716 CO₂ and immersed in Dulbecco's Modified Eagles Medium (DMEM) medium
717 (Invitrogen or HyClone™), supplemented with 10% fetal bovine serum. U251 cells
718 were plated on poly-d-lysine-coated coverslips (via submersion in 0.05 mg/mL
719 solution, Sigma-Aldrich) at 10% confluence 24 hours prior to transfection. The
720 pCAG-TDTOMATO, pCAG-H2B-GFP-2A-MyrTDTOMATO, pCAG-DCC:TDTOMATO
721 and pCAG-DCC^{kanga}:TDTOMATO plasmids (1 µg) were transfected into the plated
722 U251 cells using FuGENE 6 (Promega) in Opti-MEM (Gibco, Life Technologies).
723 Cells were then grown for 20 hours and either fixed with 4% paraformaldehyde/4%
724 sucrose or stimulated with ligand. Since 100ng/mL of recombinant NTN1 is sufficient
725 to induce morphological changes in primary oligodendrocyte precursor cells
726 (Rajasekharan et al., 2009), 200ng of recombinant mouse NTN1 protein (R&D
727 Systems) was diluted in sterile PBS and added to cultures within 2 mL media. When
728 ligand was added, cells were grown for a further 12 hours before fixation with 4%
729 PFA/4% sucrose. N2A cells were cultured and transfected as outlined for the U251
730 cells except that after NTN1 stimulation, cells were cultured for only 8 hours before
731 fixation. The pCAG-DCC:TDTOMATO wildtype and missense mutant receptor
732 constructs (1.764 µg) were also transfected into HEK293T cells cultured on acid-
733 washed coverslips using FuGENE HD (Promega) in Optimem (Gibco, Life
734 Technologies). After 24 hours, cells were fixed with 4% PFA/4% sucrose.

735

736 *NTN1-binding assay*

737 Supernatant containing alkaline phosphatase-conjugated NTN1 (NTN1-AP)
738 was generated from expression in HEK293T cells as previously described (Zelina et

739 al., 2014). The pCAG-DCC:TDTOMATO wildtype and missense mutant receptor
740 constructs (0.2 µg) were transfected into COS-7 cells using Lipofectamine® 2000
741 (Invitrogen). After 48 hours, cells were incubated with *NTN1*-AP supernatant (1:50)
742 for 90 minutes at room temperature. Cells were washed and *NTN1*-binding activity
743 was determined using colorimetric detection as previously described (Zelina et al.,
744 2014).

745

746 *Western blot*

747 Whole-cell protein extracts were prepared from N2A and U251 cells, 20 hours after
748 transfection with pCAG-DCC:TDTOMATO and pCAG-myr-TDTOMATO constructs
749 (1µg) as previously described (Bunt et al., 2010). Moreover, COS-7 protein extracts
750 were prepared 48 hours after transfection with the pCAG-DCC:TDTOMATO wildtype
751 and missense mutant receptor constructs (0.2 µg). Western blots were performed to
752 detect mouse DCC expression levels using a goat polyclonal anti-DCC antibody
753 (1:200 COS-7 or 1:800, sc-6535, Santa Cruz Biotechnology), and mouse NTN1
754 using a goat polyclonal antibody (1:500 U251 or 1:1000 N2A, AF1109, R&D
755 Systems) . GADPH was used as a loading control and was detected using rabbit
756 monoclonal anti-GADPH antibodies (1:2000, 2118, Cell Signaling Technology for
757 COS-7; 1:1000, IMG-5143A, IMGENEX for N2A and U251).

758

759 *Immunohistochemistry*

760 Brain sections were processed for standard fluorescence immunohistochemistry as
761 previously described (Moldrich et al., 2010) with the following minor modifications: All
762 sections were post-fixed on slides with 4% PFA and then subjected to antigen
763 retrieval (125°C for 4 minutes at 15 psi in sodium citrate buffer) prior to incubation
764 with primary antibodies. Alexa Fluor IgG (Invitrogen), horseradish peroxidase-
765 conjugated (Millipore) or biotinylated (Jackson Laboratories) secondary antibodies,
766 used in conjunction with Alexa Fluor 647-conjugated Streptavidin (Invitrogen)
767 amplification were used according to the manufacturer's instructions. EdU labeling
768 was performed using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen). Cell
769 nuclei were labeled using 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI,
770 Invitrogen) and coverslipped using ProLong Gold anti-fade reagent (Invitrogen) as
771 mounting media. Primary antibodies used for immunohistochemistry were: rabbit
772 anti-APC (1:250, ab15270, Abcam), mouse anti-α-dystroglycan (1:250, clone

773 I1H6C4, 05-593, Merck), rabbit anti- β -catenin (1:500, 9562, Cell signaling technology),
774 mouse anti- β -dystroglycan (1:50, MANDAG2, 7D11, Developmental studies
775 hybridoma bank), chicken anti β -galactosidase (1:500, ab9361, Abcam), rabbit anti-
776 cleaved-caspase3 (1:500, 9661, cell signaling technology), goat anti-DCC (1:500, sc-
777 6535, Santa cruz biotechnology), mouse anti-Gap43 (1:500; MAB347, Millipore),
778 mouse anti-Gfap (1:500; MAB3402, Millipore), rabbit anti-Gfap (1:500; Z0334, Dako),
779 mouse anti-Glast (or Eaat1; 1:500; ab49643, Abcam), rabbit anti-Glast (or Eaat1;
780 1:250; ab416, Abcam), mouse anti-Ki67 (1:500; 550609, BD Pharmingen), chicken
781 anti-Laminin (1:500; LS-C96142, LSBio), rabbit anti-Laminin (1:500; L9393, Sigma),
782 mouse anti-N-cadherin (CDH2; 1:250, 610921, BD Biosciences), rat anti-Nestin
783 (NES; 1:50, AB 2235915, DSHB), chicken anti-Nestin (1:1000, ab134017, Abcam),
784 goat anti-NTN1 (1:500; AF1109, R&D Systems), mouse anti-Neurofilament (1:500;
785 MAB1621, Millipore), rabbit anti-Nfia (1:500; ARP32714, Aviva Systems Biology),
786 rabbit anti-Nfib (1:500; HPA003956, Sigma), rabbit anti-neuronal-specific- β III-tubulin
787 (1:500; ab18207, Abcam), rabbit anti-phospho p44/42 Mapk (or Erk1/2; 1:250; 9101,
788 Cell Signaling Technology), rabbit anti-SOX9 (1:500, AB5535, Merck), and goat anti-
789 TDTOMATO (1:500, ab8181-200, Sicgen). For actin staining, Alexa fluor-conjugated
790 phalloidin (A22287, Thermofisher scientific) was incubated on tissue for thirty
791 minutes in the dark as per the manufacturer's instructions, prior to the addition of
792 primary antibodies. Immunohistochemistry was performed in a similar manner for
793 cultured cells, with the following minor exceptions: HEK293T cells expressing
794 wildtype and mutant pCAG-DCC:TDTOMATO constructs were not permeabilized to
795 confirm exogenous DCC receptor localisation to the plasma membrane.

796

797 *In situ hybridization*

798 In situ hybridization was performed as previously described (Moldrich et al., 2010),
799 with the following minor modifications: Fast red (Roche) was applied to detect probes
800 with fluorescence. The *Fgf8* cDNA plasmid was a kind gift from Gail Martin,
801 University of California, San Francisco. The *Ntn1* cDNA plasmid was provided by the
802 Cooper lab. The *Mmp2* cDNA plasmid was generated by the Richards lab with the
803 following primers: forward 5' - GAAGTATGGATTCTGTCCCGAG - 3' and reverse 5'
804 - GCATCTACTTGCTGGACATCAG - 3'. The *Dcc* cDNA plasmid was generated by
805 the Richards lab with the following primers, courtesy of the Allen Developing Brain

806 Atlas: forward 5' - ATGGTGACCAAGAACAGAAGGT - 3' and reverse 5' -
807 AATCACTGCTACAATCACCACG - 3'.

808

809 *Plasmid expression constructs for cell culture and in utero electroporation*

810 A TDTOMATO fluorophore (Clontech) was subcloned into a *pCAG* backbone to
811 generate the pCAG-TDTOMATO plasmid. pCAG-H2B-GFP-2A-MyrTDTOMATO was
812 provided by Arnold Kriegstein (University of California San Francisco). The *Dcc*-
813 shRNA construct was provided by Xiong Zhiqi (Chinese Academy of Sciences,
814 Shanghai; shRNA 1355 in Zhang et al., 2018). The *Dcc*-CRISPR nickase constructs
815 were designed using the ATUM tool and obtained from ATUM to target *Dcc* exon 2
816 (*Dcc*-CRISPR 1, targeting chr18:71,954,969 - 71,955,009) and *Dcc* exon 3 (*Dcc*-
817 CRISPR 2, targeting chr18:71,826,146 - 71,826,092). *Dcc*-CRISPR 1 had the
818 maximum target score across the whole DCC coding sequence, while *Dcc*-CRISPR
819 1 had the maximum target score within exon 3 only.

820

821 To generate the pCAG-DCC:tdTomato plasmid, DCC:TDTOMATO
822 (pmDCC:TDTOMATO; provided by Erik Dent, University of Wisconsin-Madison) was
823 subcloned into the pCag-DsRed2 plasmid (Addgene, 15777, Cambridge, MA), by
824 excising DsRed2.

825 For site-directed mutagenesis, the QuickChange II Site-Directed Mutagenesis Kit
826 (Stratagene, Catalogue #200524) was used in accordance with the manufacturer's
827 instructions. The following primer pairs were used for site-directed mutagenesis:

828 p.Met743Leu: Forward 5'- GAGGAGGTGTCCAACCTCAAGATGATACAGTTTGTCTG
829 - 3', reverse 5' - CAGACAAACTGTATCATCTTGAGTTGGACACCTCCTC - 3'.

830 p.Val754Met: Forward 5' - TAATATAGCCTCTCACCATGATGTTTGGGTTGAGAGG
831 - 3', reverse 5' - CCTCTCAACCCAAACATCATGGTGAGAGGCTATATTA - 3'.

832 p.Ala893Thr: Forward 5' - ACTTGTACTIONGGTACTGGCAGAAAAGCTGGTCCT - 3',
833 reverse 5' - AGGACCAGCTTTTCTGCCAGTACCAAGTACAAGT - 3'.

834 p.Val793Glu: Forward 5' -

835 ACTAGAGTCGAGTTCTCATTATGGAATCTCCTTAAAAGCTTTCAAC -3', reverse 5'
836 - GTTGAAAGCTTTTAAGGAGATTCCATAATGAGAACTCGACTCTAGT - 3'.

837 p.Gly805Glu: Forward 5' -

838 CACTTTCGTAGAGAGGGACCTCTTCTCCGGCATTGTTGAA - 3', reverse 5' -
839 TTCAACAATGCCGGAGAAGAGGTCCCTCTCTACGAAAGTG - 3'.

840 p.Met1217Val;p.Ala1250Thr: Forward 1 5' –
841 GTTCCAAAGTGGACACGGAGCTGCCTGCGTC – 3', reverse 1 5' –
842 GACGCAGGCAGCTCCGTGTCCACTTTGGAAC – 3', forward 2 5' –
843 GTACAGGGATGGTACTCACAACAGCAGGATTACTGG – 3', reverse 2 5' -
844 CCAGTAATCCTGCTGTTGTGAGTACCATCCCTGTAC – 3'.
845 p.Val848Arg: Forward 5' – CAGCCTGTACACCTCTTGGTGGGAGCATGGGGG – 3',
846 reverse 5' – CCCCCATGCTCCCACCAAGAGGTGTACAGGCTG – 3'.
847 p.His857Ala: Forward 5' – ACCCTCACAGCCTCAGCGGTAAGAGCCACAGC – 3',
848 reverse 5' – GCTGTGGCTCTTACCGCTGAGGCTGTGAGGG- 3'.
849 p.del-P3(Kanga): Forward 5' – CCACAGAGGATCCAGCCAGTGGAGATCCACC –
850 3', reverse 5' – GGTGGATCTCCACTGGCTGGATCCTCTGTGG – 3'.

851

852 *In utero electroporation*

853 In utero electroporation was performed as previously described (Suárez et al., 2014).
854 Briefly, 2 µg/µL of *Dcc*-shRNA or *Dcc*-CRISPR were combined with 0.5 µg/µL
855 TDTOMATO and 0.0025% Fast Green dye, and then microinjected into the lateral
856 ventricles of E13 *Dcc*^{kanga} embryos. 5, 35 V square wave pulses separated by 100
857 ms were administered with 3mm paddles over the head of the embryo to direct the
858 DNA into the cingulate cortex. Embryos were collected at E18 for analysis.

859

860 *Image acquisition*

861 Confocal images were acquired as either single 0.4-0.9 µm optical sections or
862 multiple image projections of ~15-20 µm thick z-stacks using either an inverted Zeiss
863 Axio-Observer fitted with a W1 Yokogawa spinning disk module, Hamamatsu
864 Flash4.0 sCMOS camera and Slidebook 6 software, or an inverted Nikon TiE fitted
865 with a Spectral Applied Research Diskovery spinning disk module, Hamamatsu
866 Flash4.0 sCMOS camera and Nikon NIS software. Alternatively, for images of
867 HEK293T cells, a LSM 780 confocal microscope was used. For imaging of NTN1-AP
868 binding, a NanoZoomer 2.0-HT whole slide imager was used in conjunction with
869 Hamamatsu (NDP_Viewer) software. For wide-field imaging of U251 and N2A cells
870 stained for cleaved-caspase 3, Zen software (Carl Zeiss) was used to capture
871 images on a Zeiss upright Axio-Imager fitted with Axio- Cam HRc camera. Images
872 were pseudocolored to permit overlay, cropped, sized, and contrast-brightness
873 enhanced for presentation with ImageJ and Adobe Photoshop software.

874

875 *Measurements and cell quantification*

876 Measurements of IHF length were performed using ImageJ v1.51s freeware
877 (National Institutes of Health, Bethesda, USA). The length of the IHF within the
878 interhemispheric midline was determined by comparing Laminin and DAPI-staining.
879 To account for inter-brain variability, this length was then normalised to the entire
880 length of the telencephalon along the interhemispheric midline, which was measured
881 from the caudal-most point of the telencephalon to the rostral edge of cerebral
882 hemispheres.

883 Cell proliferation and cell death in *Dcc*^{kanga} MZG was automatically counted
884 using Imaris software (Bitplane) from a region of interest delineated by Glast
885 staining, and excluding the IHF within a single z-slice. Cleaved-caspase 3-positive,
886 TDTOMATO-positive N2A and U251 cells were manually counted using the cell
887 counter plugin in ImageJ v1.51s freeware (National Institutes of Health, Bethesda,
888 USA), from a 1187 x 954 µm region of interest.

889 The number of Sox9-positive cell bodies was counted manually using the Cell
890 Counter plugin in ImageJ v1.51s freeware. Cell proliferation and cell death in tissue
891 was automatically counted using Imaris software (Bitplane) from a region of interest
892 delineated by Glast staining that excluded the IHF in a single z-slice.

893 The perimeter, circularity and area of U251 and N2A cells was measured from
894 mean intensity projections of TDTOMATO images following thresholding in ImageJ
895 v1.51s freeware (National Institutes of Health, Bethesda, USA). 48-191 cells per
896 condition were analysed from 3-5 biological replicates.

897

898 *Fluorescence intensity analysis*

899 To compare fluorescence intensity, tissue sections were processed under identical
900 conditions for immunofluorescence. Fluorescent images at 20x or 40x magnification
901 were acquired using identical exposure settings for each fluorescent signal and
902 identical number of slices through the z plane. A multiple intensity projection was
903 created for each z-stack to create a 2D image. Identical regions of interest were
904 outlined in ImageJ freeware and the fluorescence intensity was plotted versus the
905 distance and the average fluorescence intensity was calculated.

906

907 *Quantification and statistical analysis*

908 A minimum of three animals were analysed for each separate phenotypic analysis.
909 Sex was not determined for embryonic studies. A mix of male and female adult mice
910 were used to determine the length of the IHF in *Dcc^{kanga}* and C57Bl/6 mice. All
911 measurements and cell counting were performed on deidentified files so the
912 researcher remained blind to the experimental conditions. For comparison between
913 two groups, the data was first assessed for normality with a D'Agostino-Pearson
914 omnibus normality test and then statistical differences between two groups were
915 determined either with a parametric Student's t-test, or a non-parametric Mann-
916 Whitney test in Prism software (v.6-v.8; GraphPad). To test whether CC or HC length
917 was correlated with IHF length as normalised to total telencephalic midline length,
918 the data was also assessed for normality with D'Agostino-Pearson omnibus
919 normality tests, and a Pearson correlation coefficient was computed if the data was
920 representative of a Gaussian distribution; otherwise, a nonparametric Spearman
921 correlation was performed. For multiple comparisons of cell culture conditions or
922 measurements of GFAP fluorescence across mouse strains, a Kruskal-Wallis test
923 was performed with post-hoc Dunn's multiple comparison test. $p \leq 0.05$ was
924 considered significantly different, where all p values are reported in text. All values
925 are presented as mean \pm standard error of the mean (SEM).

926

927 CONTACT FOR REAGENT AND RESOURCE SHARING

928 Further information and requests for resources and reagents should be directed to
929 and will be fulfilled by Professor Linda J Richards (richards@uq.edu.au).

930

931 DATA AVAILABILITY

932 Microscopy data, measurements and statistical analyses are available. This study
933 did not generate code.

934

935

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959

960 **Declaration of Interest**

961 The authors declare no competing financial interests.

962

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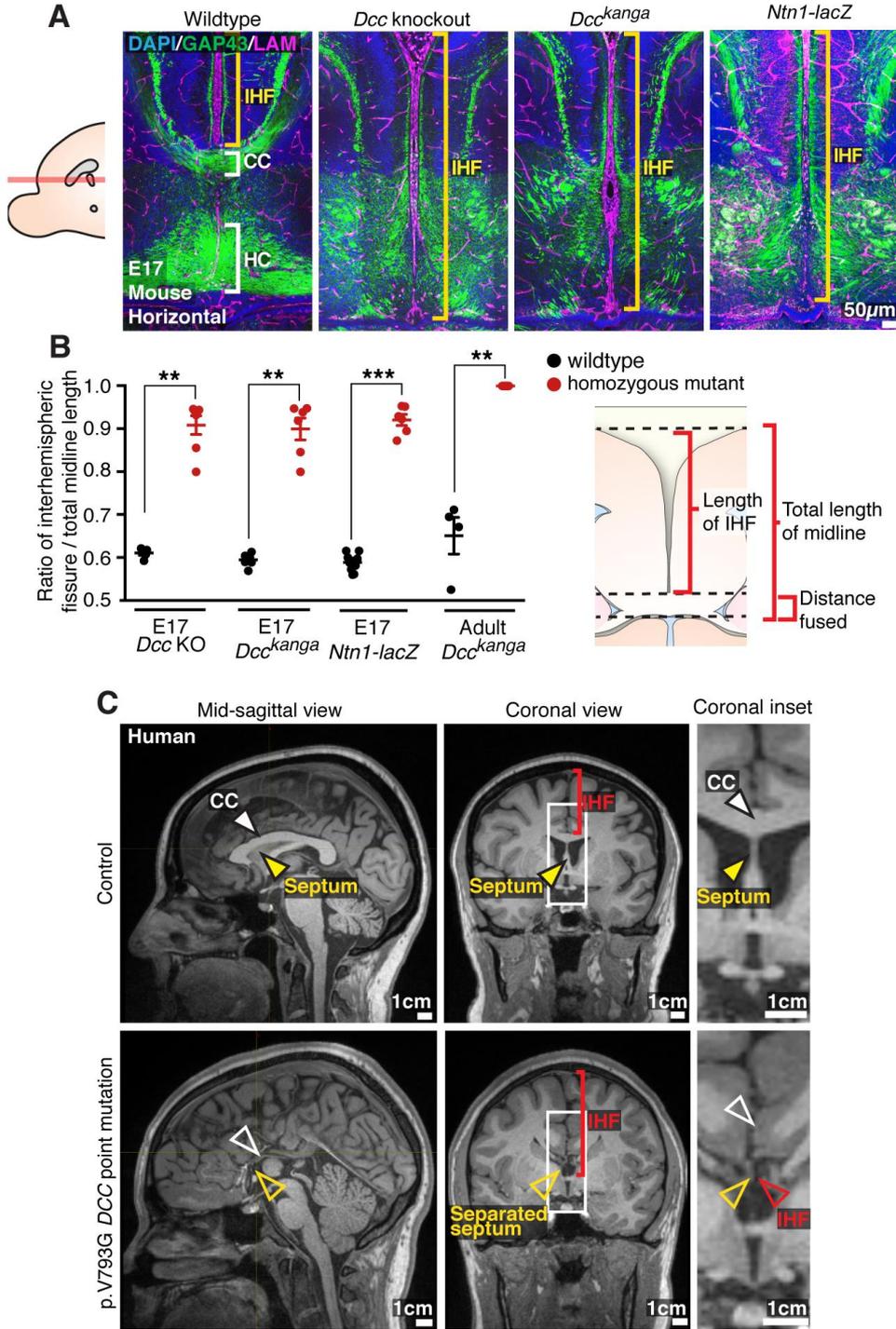
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Figures and legends



1279 **Figure 1: NTN1 and DCC are crucial for remodelling of the IHF, CC and HC**
1280 **formation**

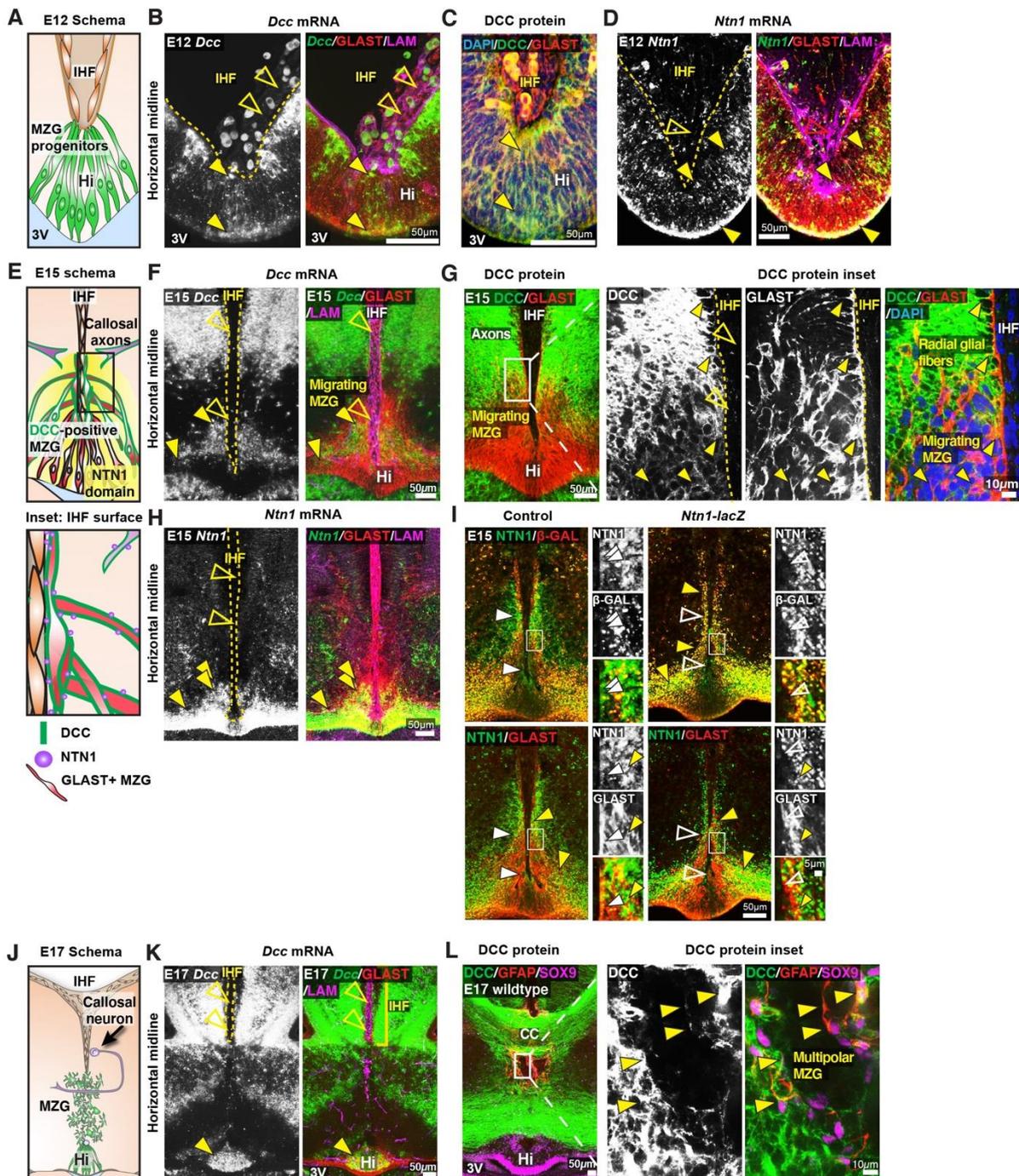
1281 (A) Staining for Gap43-positive axons (green) and pan-Laminin (LAM)-positive
1282 leptomeninges and basement membrane (magenta) in wildtype, *Dcc* knockout,
1283 *Dcc^{kanga}*, and *Ntn1-lacZ* mice at E17, indicate midline formation or absence of the CC
1284 and HC (white brackets) and extent of the IHF (yellow brackets).

1285 (B) The ratio of IHF length over the total midline length with schema.

1286 (C) T1-weighted MR images of a control subject compared with an individual with a
1287 DCC mutation demonstrate the presence or absence of the CC (white arrowheads)
1288 and extent of the IHF (red arrowheads and brackets) within the septum (yellow
1289 arrowheads).

1290 Graph represents mean \pm SEM. Statistics by Mann-Whitney test: **p < 0.01, ***p <
1291 0.001. See related Figure 1-figure supplement 1 and Supplementary File 1.

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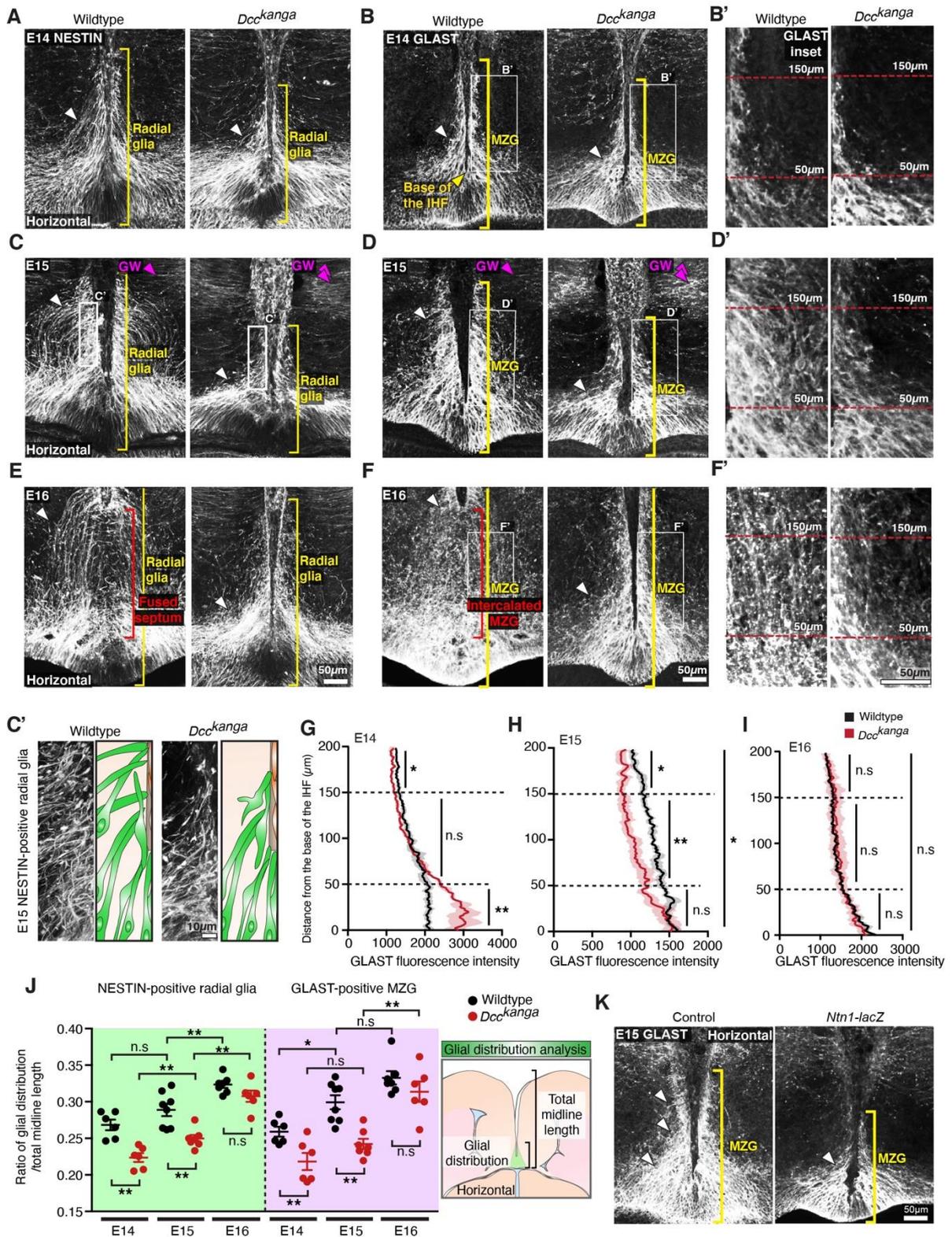
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1295 **Figure 2: DCC and NTN1 are expressed in MZG and MZG progenitors**

1296 (A, E, and J) Schematics depicting the cellular composition of the ventral
1297 telencephalic midline at E12, E15 and E17.

1298 (B, F and K) *Dcc* mRNA (green), Glast-positive glia (red), and pan-laminin (LAM)-
1299 positive leptomeninges and basement membrane (magenta) in E12, E15, and E17
1300 wildtype mice reveal *Dcc*-positive/Glast-positive glial fibers (yellow arrowheads) and
1301 absence of *Dcc* within the IHF (open yellow arrowheads).

1302 (C and G) DCC protein (green) and Glast protein (red) at E12 and E15 in wildtype
1303 mice reveal DCC-positive/Glast-positive glial fibers (yellow arrowheads) and
1304 absence of DCC within the IHF (open yellow arrowheads).
1305 (D and H) *Ntn1* mRNA (green), Glast (red) and pan-LAM (magenta) in E12 and E15
1306 wildtype mice show *Ntn1*-positive/Glast-positive glial fibers (yellow arrowheads) and
1307 absence of *Ntn1* within the IHF (open yellow arrowheads).
1308 (E inset) Schema of DCC and NTN1 expression at the E15 IHF surface, based on
1309 the results from F-I and Figure 2-figure supplement 1.
1310 (I) NTN1 (green) and Glast (red) or β -galactosidase (β -GAL; red) immunolabelling in
1311 E15 control and *Ntn1-lacZ* mice identify regions of NTN1 staining present in control
1312 heterozygotes and absent in homozygous *Ntn1-lacZ* mice (white arrowheads) and
1313 NTN1-/ β -GAL-positive puncta located in Glast-positive glia (yellow arrowheads), with
1314 insets.
1315 (L) DCC protein (green), glial-specific nuclear marker SOX9 (magenta) and mature
1316 astroglial marker (GFAP) in E17 wildtype mice identify DCC-positive/GFAP-
1317 positive/SOX9-positive glia (yellow arrowheads). 3V = third ventricle, Hi =
1318 telencephalic hinge, See related Figure 2-figure supplement 1.
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Figure 3: NTN1 and DCC regulate MZG morphology and spatial distribution

Nestin-positive radial glia (white; A, C and E) and Glast-positive glia (white; B, D, F and K) in E14 - E16 *Dcc^{kanga}* mice (A-F) and E15 *Ntn1-LacZ* mice (K) demonstrate the distribution of glial processes along the IHF surface (yellow brackets) and lateral

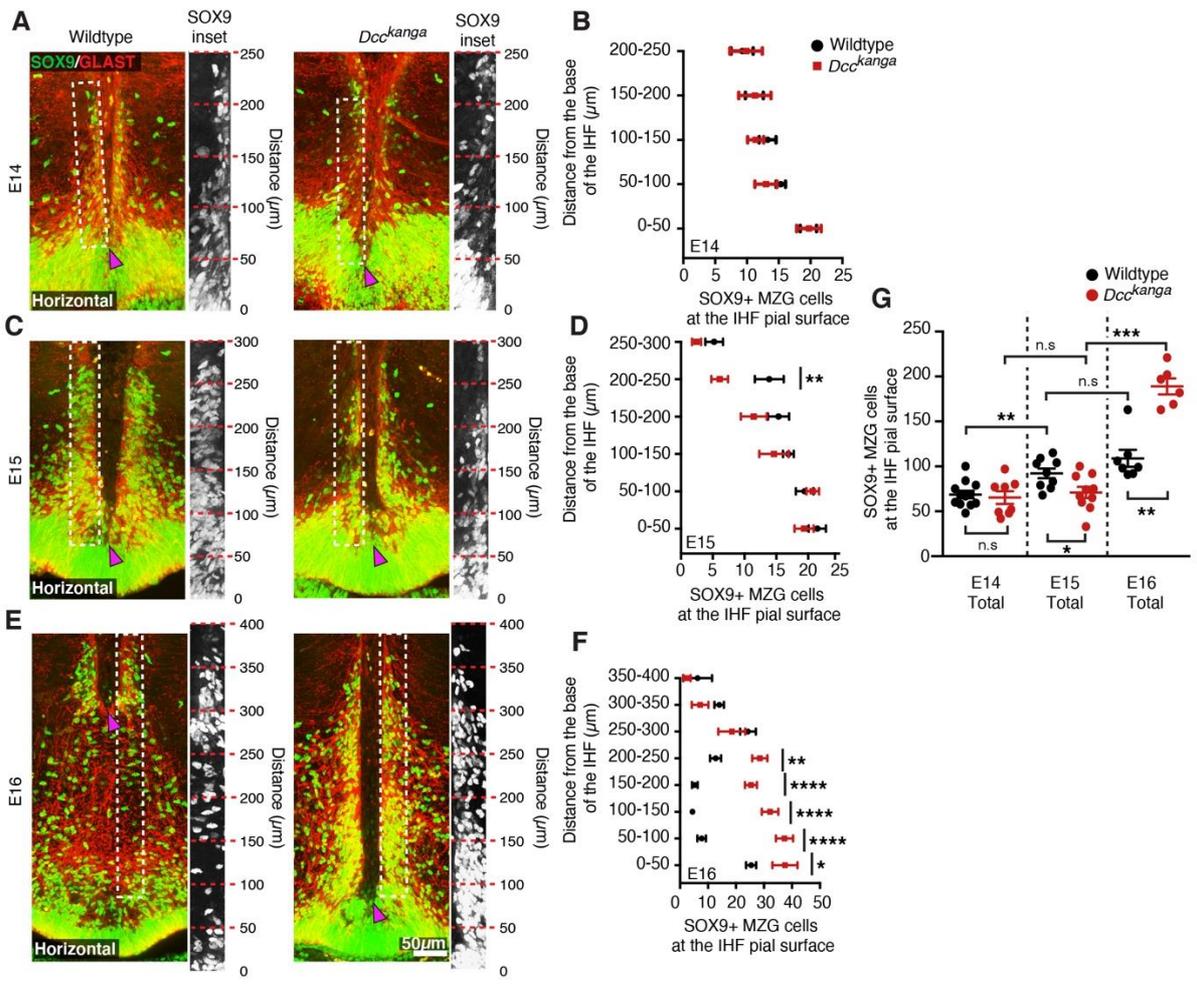
1326 to the IHF (white arrowheads) with insets (C', B', D' and F'). Radial fibers of the glial
1327 wedge (GW) are indicated with magenta arrowheads.

1328 The mean fluorescence intensity of Glast staining between wildtype and *Dcc^{kanga}*
1329 mice at E14 (G), E15 (H) and E16 (I) based on the results from B, D and F,
1330 respectively.

1331 (J) The ratio of glial distribution over total midline length, with schema, based on the
1332 results from A-F.

1333 All graphs represent mean \pm SEM. Statistics by Mann-Whitney test (C), or a Two-
1334 way ANOVA test with post Sidak's multiple comparison test (D): n.s = not significant,
1335 *p < 0.05, **p < 0.01. See related Figure 3-figure supplement 1 and Supplementary
1336 File 1.

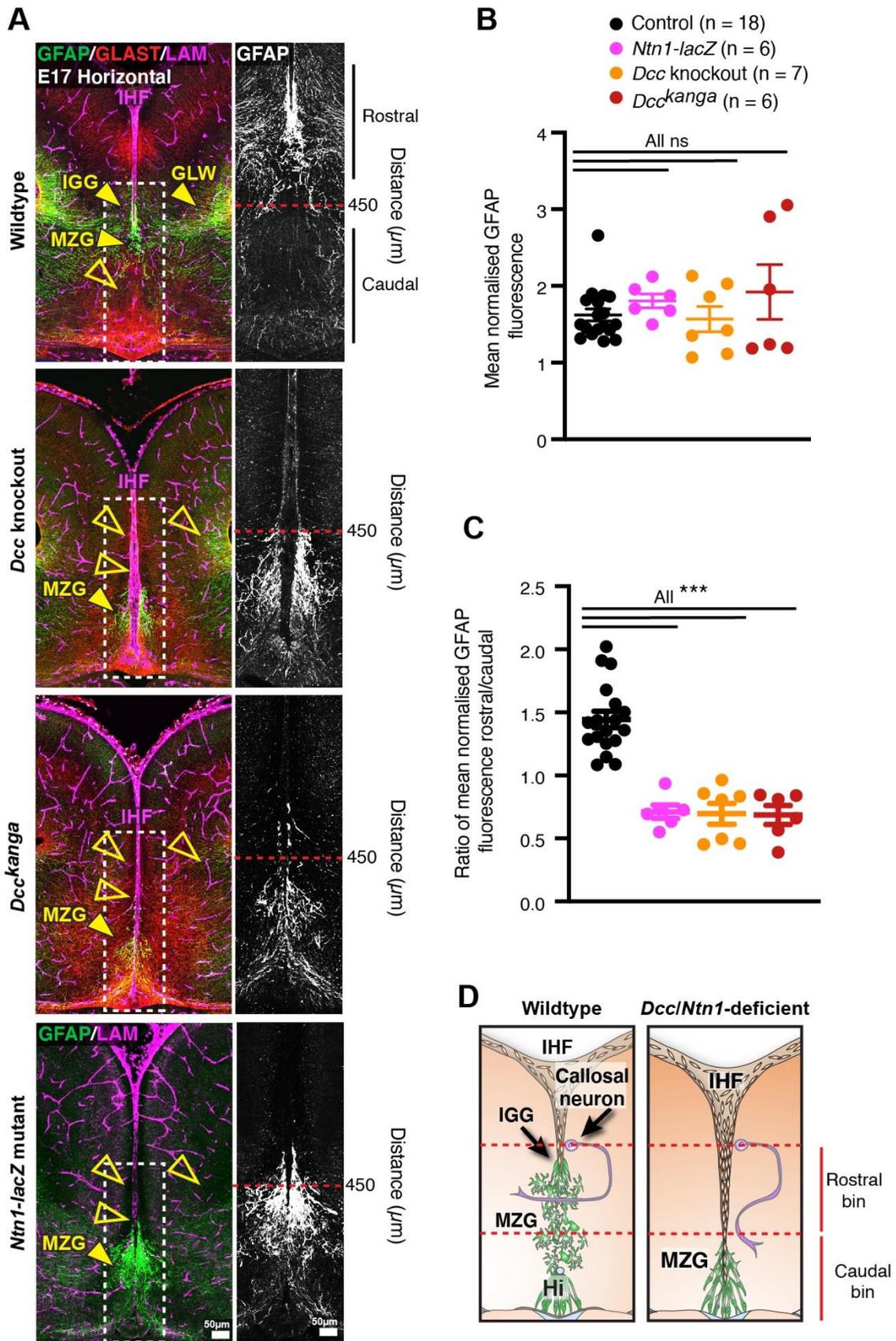
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Figure 4: DCC regulates MZG migration to the IHF surface

(A, C, E) Nuclear glial marker SOX9 (green) and MZG marker Glast (red), in E14-
 E16 *Dcc^{kanga}* mice reveal SOX9-positive/Glast-positive MZG at the pial IHF surface
 (boxed region and insets) above the base of the IHF (magenta arrowhead).
 (B, D, F, G) Quantification of SOX9-positive/Glast-positive MZG at the IHF pial
 surface based on the results from A, C and E.
 All graphs represent mean ± SEM. Statistics by Mann-Whitney test (E) or Two-way
 ANOVA with post Sidak's multiple comparison test (B-D): *p < 0.05, **p < 0.01, ***p
 < 0.001, ****p < 0.0001, n.s = not significant. See related Figure 4-figure supplement
 1 and Supplementary File 1.



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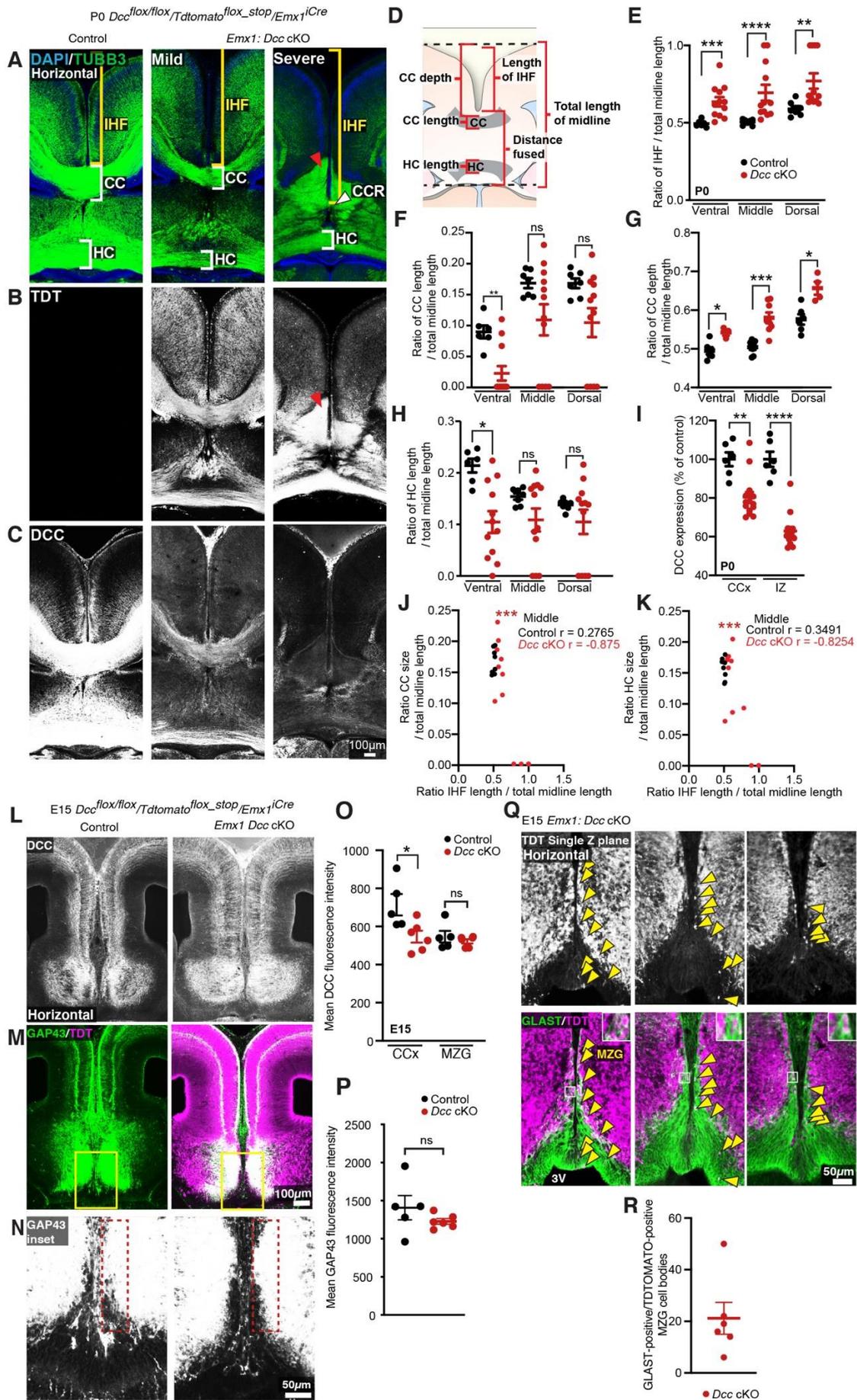
Figure 5: NTN1 and DCC regulate MZG organisation during IHF remodelling

1354 (A) Gfap-positive mature astroglia (green or white in inset), Glast-positive glia (red),
1355 and pan-Laminin (LAM)-positive IHF and basement membrane (magenta) in E17
1356 wildtype *Dcc*^{kan^{ga}}, *Dcc* knockout, and *Ntn1-LacZ* mice. Yellow arrowheads indicate
1357 presence (filled) or absence (open) of midline glial populations, the midline zipper
1358 glia (MZG), indusium griseum glia (IGG) and glial wedge (GW). Fluorescence
1359 intensity of Gfap staining from insets or bins in insets (red dotted line) was quantified
1360 in B and C.

1361 (D) Schema of MZG development, IHF remodelling and CC formation in wildtype
1362 mice and mice deficient for NTN1 or DCC. Red dotted lines indicate rostral and
1363 caudal bins that were used to calculate the ratio of GFAP fluorescence in C.

1364 All graphs represent mean \pm SEM. Statistics by Kruskal-Wallis test with post-hoc
1365 Dunn's multiple comparison test. ***p < 0.001, n.s = not significant. See related
1366 Figure 4-figure supplement 1, Figure 5-figure supplement 5 and Supplementary File
1367 1.

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1370 **Figure 6: Conditional knockdown of DCC within EMX1 cells causes a spectrum**
1371 **of callosal phenotypes**

1372 (A) Axonal marker TUBB3 (green), (B) TDT (white) or (C) DCC (white) in P0 *Dcc*
1373 cKO demonstrate a spectrum of callosal and IHF remodelling phenotypes and a
1374 reduction in DCC expression within mice expressing *Emx1^{iCre}*. The CC or CC
1375 remnant (CCR) and HC are indicated with white brackets or white arrowheads, and
1376 the IHF is indicated with yellow brackets. Red arrowheads indicate axon bundles that
1377 have not crossed the midline.

1378 (D) Schema of measurements taken for quantification shown in C-E.

1379 (E) Quantification of the ratio of IHF length normalised to total telencephalic midline
1380 length measured for P0 *Dcc* cKO mice.

1381 (F and G) Quantification of CC length (F) and depth (G) normalised to the total
1382 telencephalic midline length in P0 *Dcc* cKO mice.

1383 (H) Quantification of HC length normalised to the total telencephalic midline length in
1384 P0 *Dcc* cKO mice.

1385 (I) Quantification of DCC expression measured from the cingulate cortex (CCx) and
1386 intermediate zone (IZ) of *Dcc* cKO mice.

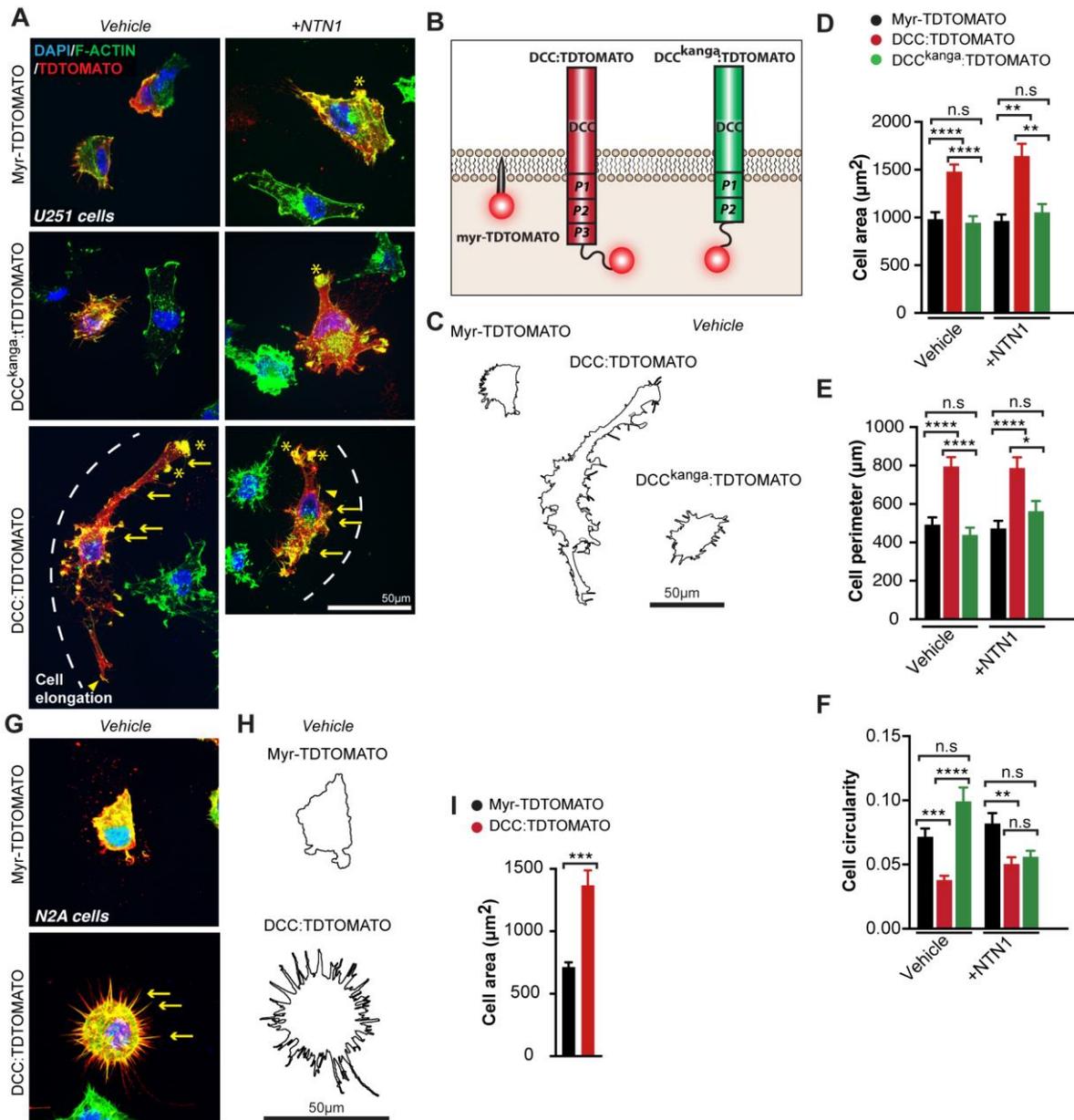
1387 (J and K) Scatterplots of the relationship between CC length (J) or HC length (K)
1388 normalised to total telencephalic midline length and IHF length normalised to total
1389 telencephalic midline length for middle horizontal sections of P0 *Dcc* cKO mice.
1390 Pearson r correlations are shown.

1391 (L) DCC (white), (M and N) axonal marker GAP43 (green or white, insets) and TDT
1392 (magenta) in E15 *Dcc* cKO mice, with quantification of mean DCC fluorescence in
1393 (O), and quantification of mean GAP43 fluorescence within 50 µm from the IHF
1394 (dotted red lines) in (P) .

1395 (Q) TDT (white or magenta) and glial marker GLAST (green) in E15 *Dcc* cKO with
1396 insets and yellow arrowheads indicating GLAST-positive/TDT-positive MZG, and
1397 quantified in R.

1398 All graphs represent mean ± SEM. Statistics by Mann-Whitney test or unpaired t test:
1399 *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s = not significant. See related
1400 Figure 5-figure supplement 1 and Supplementary File 1.

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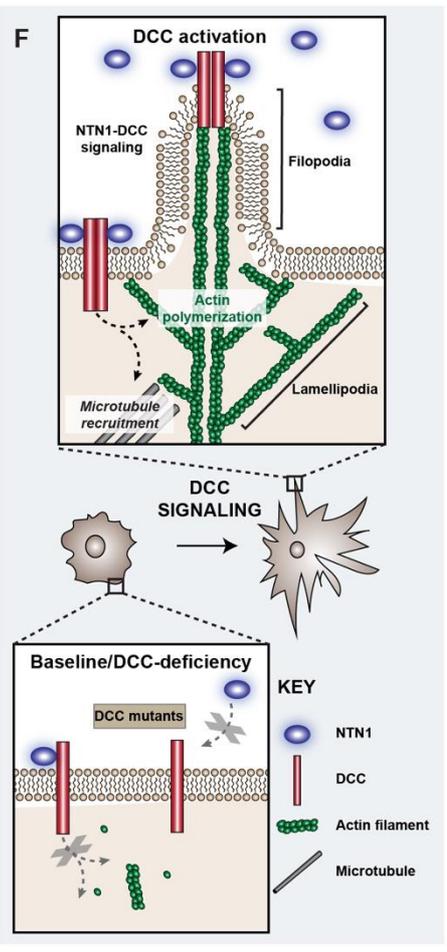
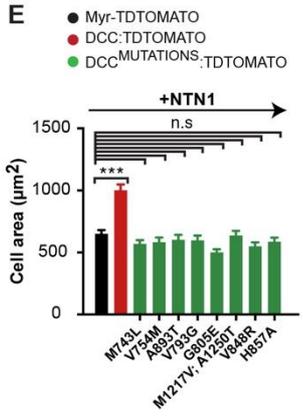
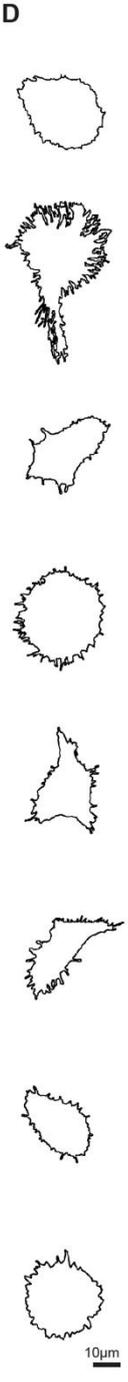
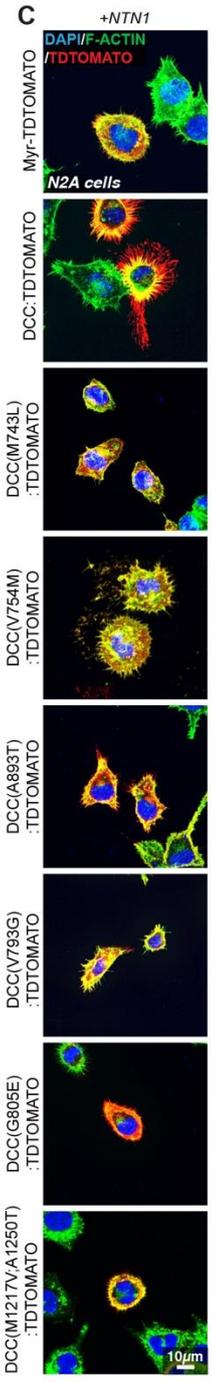
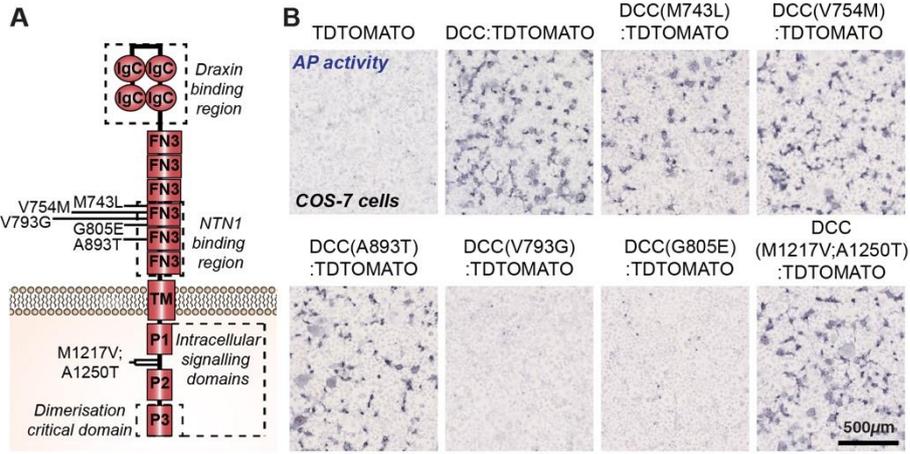
1404 **Figure 7: NTN1-DCC signalling promotes cytoskeletal remodelling of astroglia**

1405 (A, G) Representative images of U251 glioblastoma cells (A) and N2A cells (G)
 1406 immunolabelled for TDTOMATO (red), and F-actin (green) following transfection with
 1407 plasmids encoding Myr-TDTOMATO, DCC:TDTomato, or DCC^{kanga}:TDTomato
 1408 demonstrating the presence of actin-rich regions resembling filopodia (yellow
 1409 arrows), lamellipodia (yellow arrowheads) and membrane ruffles (yellow asterisks)
 1410 with/without stimulation with recombinant mouse NTN1 protein.

1411 (B) Schema of predicted structure of proteins on the cell membrane encoded by the
 1412 plasmids expressed in cells from A and G.

1413 (C and H) Outline of cell perimeter generated from images in A and G respectively.

1414 (D-F and I) Quantification of the area, perimeter and circularity of cells represented in
1415 A and G. Graphs represent mean \pm SEM. Statistics by Kruskal-Wallis test for
1416 multiple comparisons: n.s = not significant, *p < 0.05, **p < 0.01, ***p < 0.001,
1417 ****p<0.0001. See related Figure 6-figure supplement 1 and Supplementary File 1.
1418



1420 **Figure 8: DCC mutations associated with human callosal agenesis are unable**
1421 **to modulate cell shape and show varied NTN1 binding**

1422 (A) Schema of transmembrane receptor DCC and its structural domains. Lines
1423 indicate the position of altered residues from missense *DCC* pathogenic variants
1424 identified in human individuals with CC abnormalities. FN3 = fibronectin type III-like
1425 domain, IgC = immunoglobulin-like type C domain, TM = transmembrane domain, P
1426 = P motif.

1427 (B) Colourimetric detection of alkaline phosphatase activity in COS-7 cells
1428 transfected with plasmids encoding TDTOMATO, DCC:TDTOMATO, and mutant
1429 DCC:TDTOMATO constructs, and incubated with a NTN1 alkaline phosphatase
1430 fusion protein.

1431 (C) Representative images of N2A cells immunolabelled for TDTOMATO (red), and
1432 F-actin (green) following transfection with plasmids encoding Myr-TDTOMATO,
1433 DCC:TDTOMATO, or DCC:TDTOMATO carrying missense mutations and stimulated
1434 with recombinant mouse NTN1 protein.

1435 (D) Outline of cell perimeter generated from images in B.

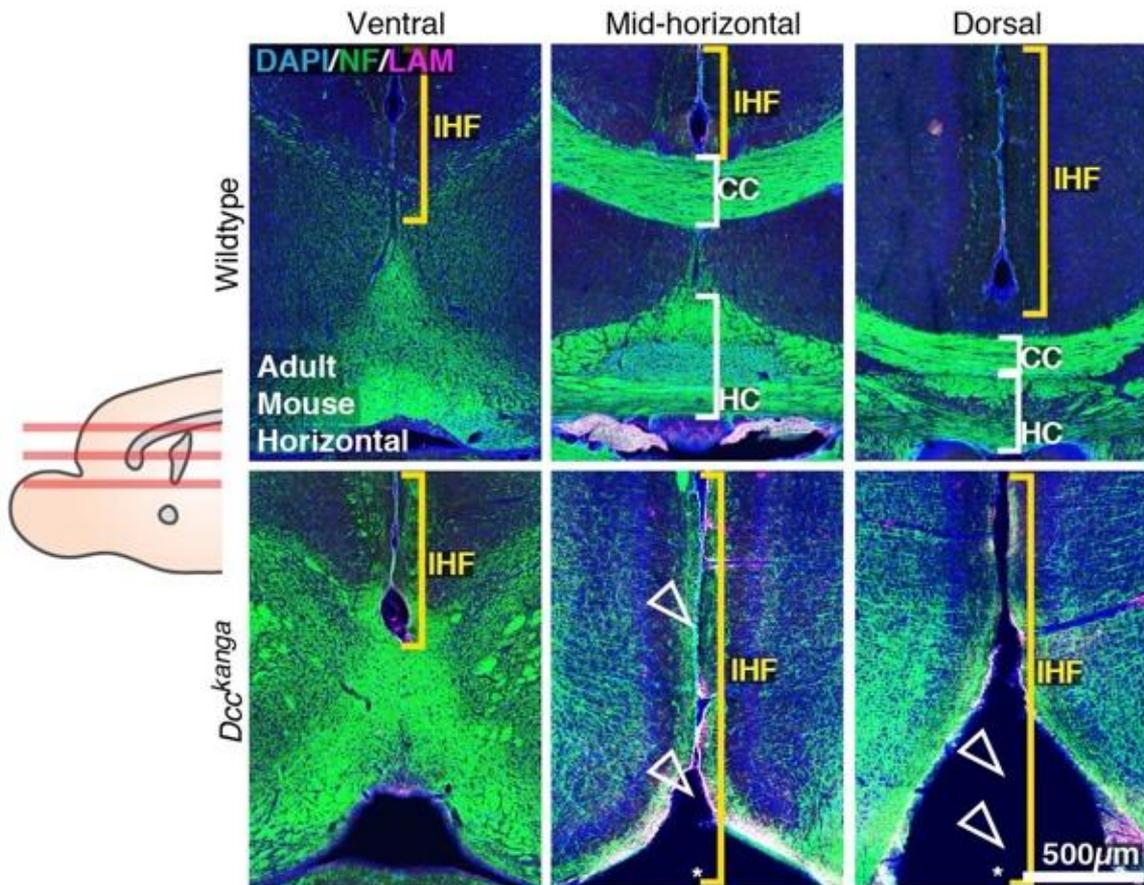
1436 (E) Quantification of the area of cells represented in B. Graph represents mean \pm
1437 SEM. Statistics by Kruskal-Wallis test for multiple comparisons: n.s = not significant,
1438 *** $p < 0.001$.

1439 (F) Schema of model for DCC-mediated changes in cell shape: Activation of DCC by
1440 NTN1 induces dimerisation of the receptor and recruits intracellular signaling
1441 effectors to regulate actin polymerisation for filopodia and lamellipodia formation, and
1442 to regulate microtubule dynamics to promote membrane protrusions. Mutations that
1443 affect DCC signalling prevent DCC-mediated changes in cell shape.

1444 See related Figure 6-figure supplement 1 and Supplementary File 1.

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1448 **Figure 1-figure supplement 1: The IHF is not remodelled in adult *Dcc^{kanga}* mice**

1449 Neurofilament (NF)-positive axons (green) and pan-Laminin (LAM)-positive

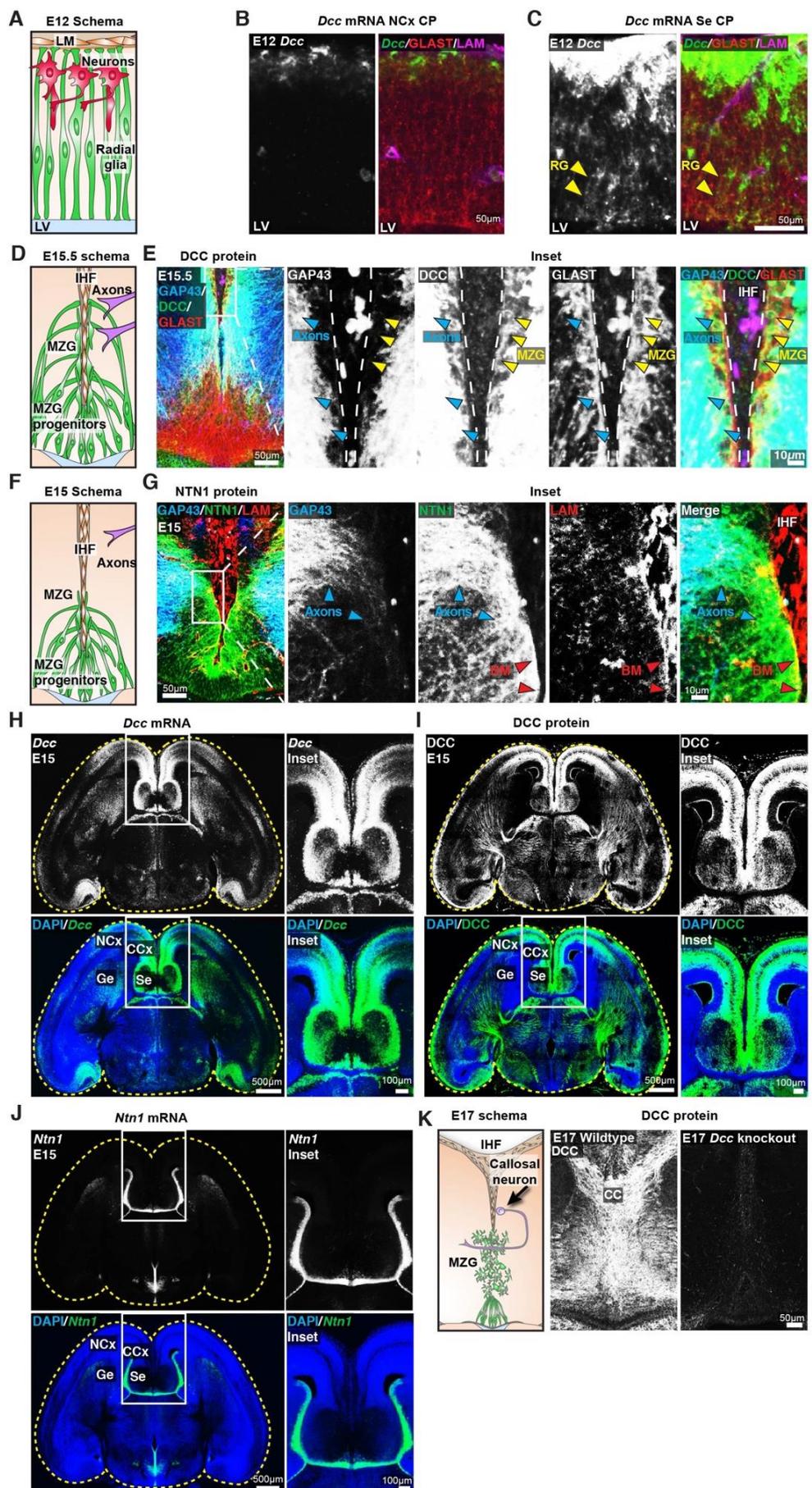
1450 leptomeninges and basement membrane (magenta) in adult wildtype and *Dcc^{kanga}*

1451 mice reveal presence/absence of the CC and HC (white brackets and arrowheads),

1452 the extent of the IHF (yellow brackets) and absence of the septal substrate in

1453 *Dcc^{kanga}* mice (asterisks).

1454



1456 **Figure 2-figure supplement 1: DCC is expressed in MZG**

1457 (A, D and F) Schemas of key cellular components within the telencephalic midline.

1458 (B and C) *Dcc* mRNA (green), Glast-positive glia (red), and pan-laminin (LAM)-
1459 positive leptomeninges and basement membrane (magenta) across the cortical plate
1460 (Cp) within the neocortex (NCx) or septum (Se) in horizontal sections of wildtype
1461 mice reveals *Dcc*-positive/Glast-positive radial glial (RG) fibers (yellow arrowheads).
1462 LV = lateral ventricle.

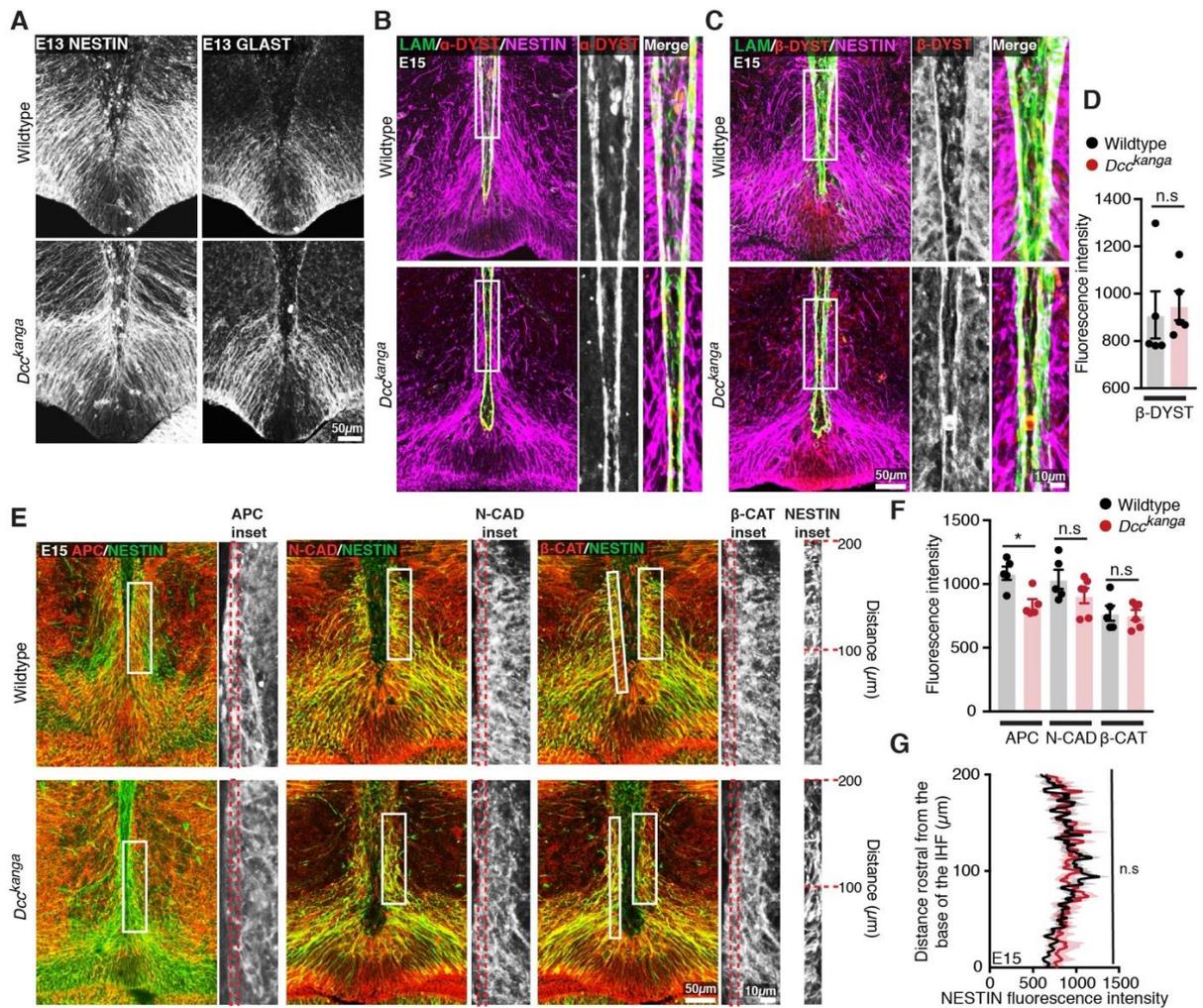
1463 (E) DCC protein (green), Gap43-positive axons (blue), and Glast-positive MZG (red)
1464 in horizontal sections of E15 wildtype mice (right panels), indicate DCC-
1465 positive/Glast-positive cells (yellow arrowheads) and DCC-positive/Gap43-positive
1466 axons (blue arrowheads) that are approaching the midline and are adjacent to MZG.

1467 (G) Gap43-positive axons (blue), NTN1 protein (green), and pan-Laminin (LAM)-
1468 positive leptomeninges and basement membrane (red) in horizontal sections of E15
1469 wildtype mice reveal NTN1-positive/Gap43-positive axons (blue arrowheads)
1470 approaching the midline and NTN1-positive/LAM-positive basement membrane (BM;
1471 red arrowheads) of the IHF.

1472 (H, I and J) Mid-horizontal tissue sections encompassing the entire telencephalon
1473 (yellow outlines) with in situ hybridization for *Dcc* mRNA or *Ntn1* mRNA or
1474 immunohistochemistry for DCC protein (all white or green), counterstained with DAPI
1475 (blue). Insets of the telencephalic midline are shown on the right.

1476 (K) DCC immunohistochemistry in horizontal sections of E17 wildtype and *Dcc*
1477 knockout mice with schema of key cellular components within the telencephalic
1478 midline.

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Figure 3-figure supplement 1: DCC is not required for endfeet attachment or molecular polarity of MZG

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1483 (A) Nestin-positive radial glia (white), and Glast-positive MZG (white) in horizontal
1484 sections of E13 wildtype and *Dcc^{kanga}* mice.

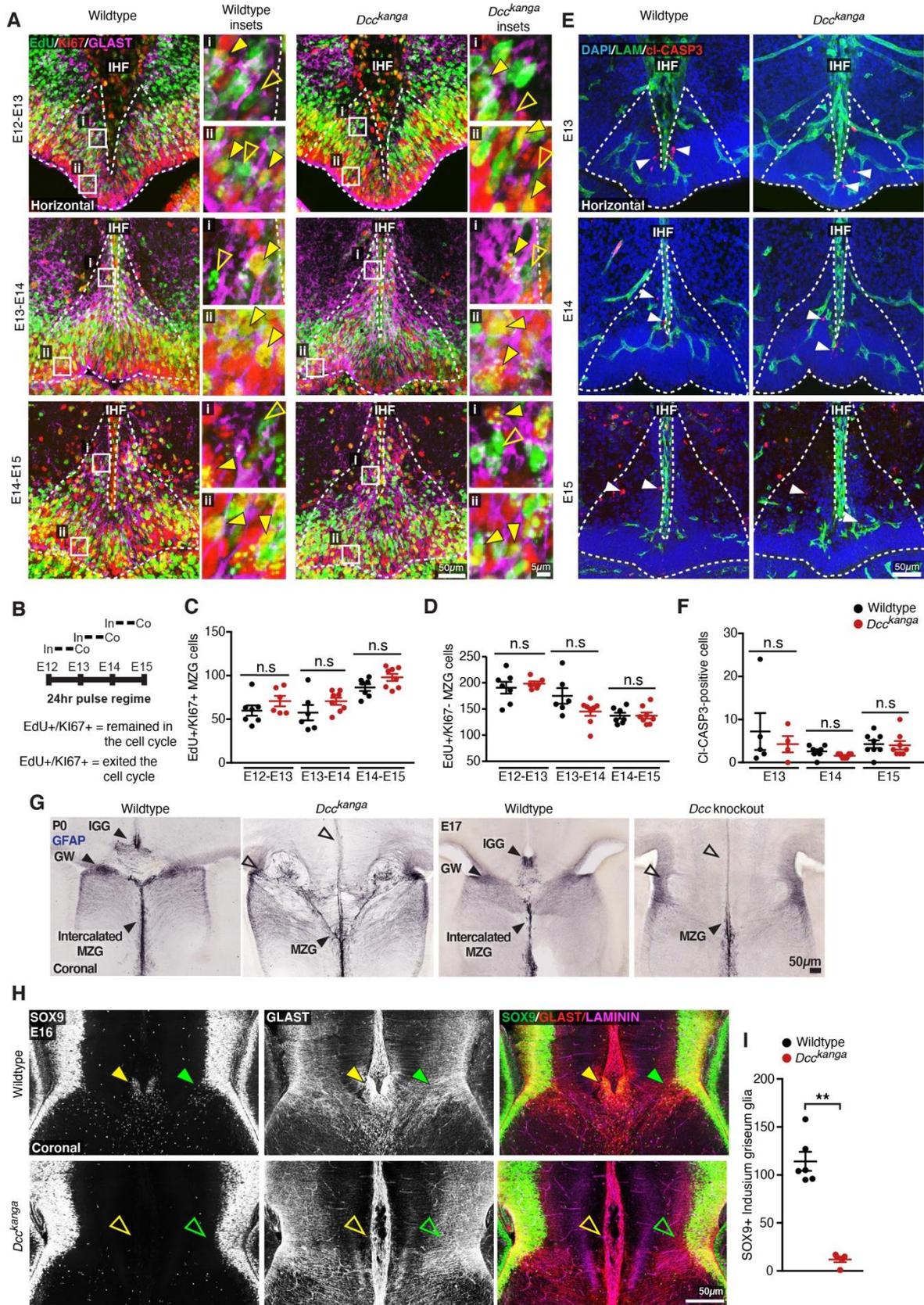
1485 (B and C) Pan-Laminin (LAM)-positive leptomeninges and basement membrane
1486 (green), Nestin-positive radial glia (magenta), and α -dystroglycan (α -DYST; red; B),
1487 or β -dystroglycan (β -DYST; red, C) in horizontal sections of E15 wildtype and
1488 *Dcc^{kanga}* mice.

1489 (D) Quantification of fluorescence intensity of β -DYST along 200 μ m of the IHF
1490 surface as outlined with red dotted box in C.

1491 (E) Nestin-positive radial glia (green) with either Adenomatous polyposis coli (APC,
1492 red), N-cadherin (N-CAD; red), or β -catenin (β -CAT; red) in horizontal sections of
1493 E15 wildtype and *Dcc^{kanga}* mice with insets.

1494 (F) Quantification of the fluorescence intensity of APC, N-CAD and β -CAT within 5
1495 μ m of the IHF as outlined in red dotted-edged boxes from E.

1496 (G) Quantification of the fluorescence intensity of Nestin-positive radial glial endfeet
1497 within 5 μm of the IHF surface from inset in E.
1498 All graphs represent mean \pm SEM. Statistics by Mann-Whitney test: n.s = not
1499 significant, * $p < 0.05$. See related Supplementary File 1.
1500



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1502

1503 **Figure 4-figure supplement 1: DCC does not regulate the proliferation or cell**
1504 **death of MZG but regulates the formation of the indusium griseum glia and**
1505 **glial wedge**

1506 (A) Mouse MZG cells were birth-dated with the thymidine analog EdU every 24
1507 hours, from E12 to E15 in wildtype and *Dcc^{kanga}* mice. Representative images of EdU
1508 (green), cell cycle marker, Ki67 (red), and MZG marker Glast (magenta) are shown
1509 for wildtype and *Dcc^{kanga}* mice, with the distribution of MZG progenitors within the
1510 telencephalic hinge niche outlined with white dotted lines. Yellow arrowheads in
1511 insets point out EdU cells that are either Ki67-positive (filled arrowheads) or Ki67-
1512 negative (open arrowheads) in selected insets. The number of cells expressing each
1513 marker is quantified in (C) and (D).

1514 (B) Schema of the EdU injection (In) and collection (Co) regime and interpretation of
1515 co-labelled and non-co-labelled cells.

1516 (E) Laminin(LAM)-positive leptomeninges and basement membrane (green) and
1517 cleaved-caspase3-positive apoptotic cells (red, white arrowheads) in E13-E15
1518 wildtype and *Dcc^{kanga}* mice. The number of cleaved-caspase3 (Cl-CASP3)-positive
1519 cells within the telencephalic hinge niche (white dotted lines) is quantified in (F).

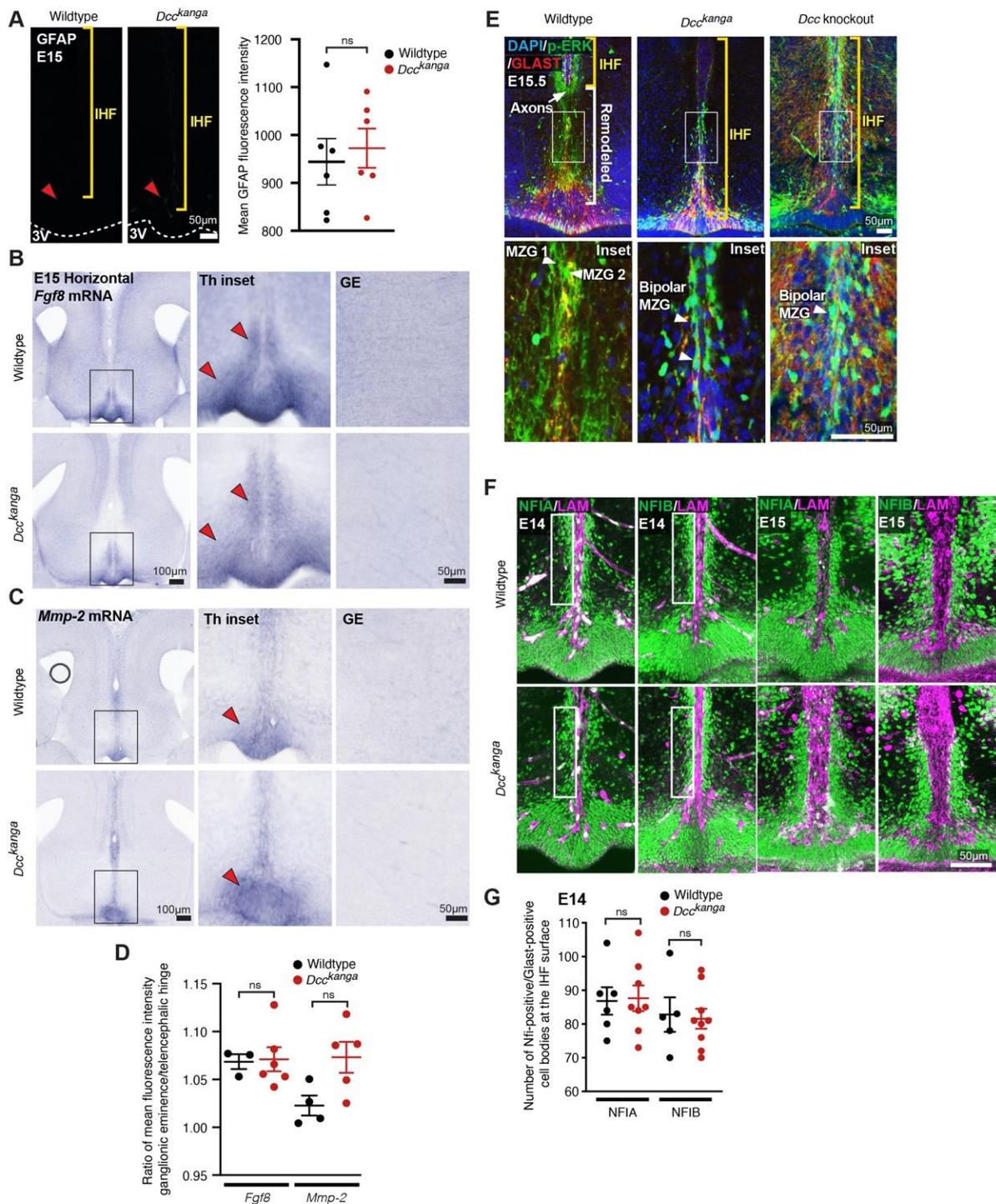
1520 (G) Mature astroglial marker GFAP in coronal sections of P0 *Dcc^{kanga}* mice and E17
1521 *Dcc* knockout mice and their wildtype littermates reveals midline glial populations,
1522 the glial wedge (GW), the indusium griseum glia (IGG), and the MZG (filled
1523 arrowheads) or their absence/malformation (open arrowheads).

1524 (H) Glial-specific cell body marker SOX9 (white or green), glial cell membrane
1525 marker Glast (white or red), and IHF marker Laminin (magenta) in E16 coronal
1526 sections from *Dcc^{kanga}* mice indicate the presence or absence of SOX9-
1527 positive/Glast-positive cell bodies at the pial surface of the IHF (yellow arrowheads)
1528 and within the intermediate zone (green arrowheads).

1529 (I) Quantification of SOX9-positive IGG cell bodies at the pial surface of the IHF in
1530 E16 wildtype and *Dcc^{kanga}* mice from immunohistochemistry in G.

1531 All graphs represent mean \pm SEM. Statistics by Mann-Whitney test: n.s = not
1532 significant, **p < 0.01. See related Supplementary File 1.

1533



1535 **Figure 5-figure supplement 1: DCC is not required for astroglial differentiation**
 1536 **of MZG**

1537 (A) Mature astroglial marker Gfap (white) in horizontal sections of E15 wildtype and
 1538 *Dcc^{kanga}* mice with quantification of Gfap average fluorescence intensity. The surface
 1539 of the third ventricle (3V) is outlined with dotted white lines. Red arrowheads indicate

1540 reactive blood vessels that are not Gfap-positive glia. Yellow brackets indicate the
1541 position of the interhemispheric fissure (IHF).
1542 *Fgf8* mRNA (B) or *Mmp-2* mRNA (C) in horizontal sections of E15 wildtype and
1543 *Dcc^{kanga}* mice. Red arrowheads indicate reactivity in the telencephalic hinge (Th) in
1544 insets, right. A region of the ganglionic eminence (GE) where *Fgf8* is not expressed
1545 is shown and was used to normalise specific *Fgf8* expression within the Th with
1546 background immunoreactivity as quantified in D.
1547 (E) Phosphorylated p44/42 Mapk or Erk1/2 (p-ERK, green), and Glast-positive MZG
1548 (red) in horizontal sections of E15.5 wildtype, *Dcc^{kanga}* mice and *Dcc* knockout mice
1549 reveal extent of IHF (yellow brackets) and remodelled regions of the septum (white
1550 brackets) with p-ERK-positive MZG in insets.
1551 (F) Nuclear factor I (NFI) A or B (green), and pan-Laminin (LAM)-positive
1552 leptomeninges and basement membrane (magenta) in horizontal sections of E14
1553 and E15 wildtype and *Dcc^{kanga}* mice. NFI-positive/Glast-positive MZG cell bodies at
1554 the IHF surface are outlined with white boxes and quantified in G. Data is
1555 represented as mean \pm SEM. Significant differences were determined with non-
1556 parametric Mann-Whitney tests. ns = not significant, Hi = telencephalic hinge. LAM =
1557 laminin
1558

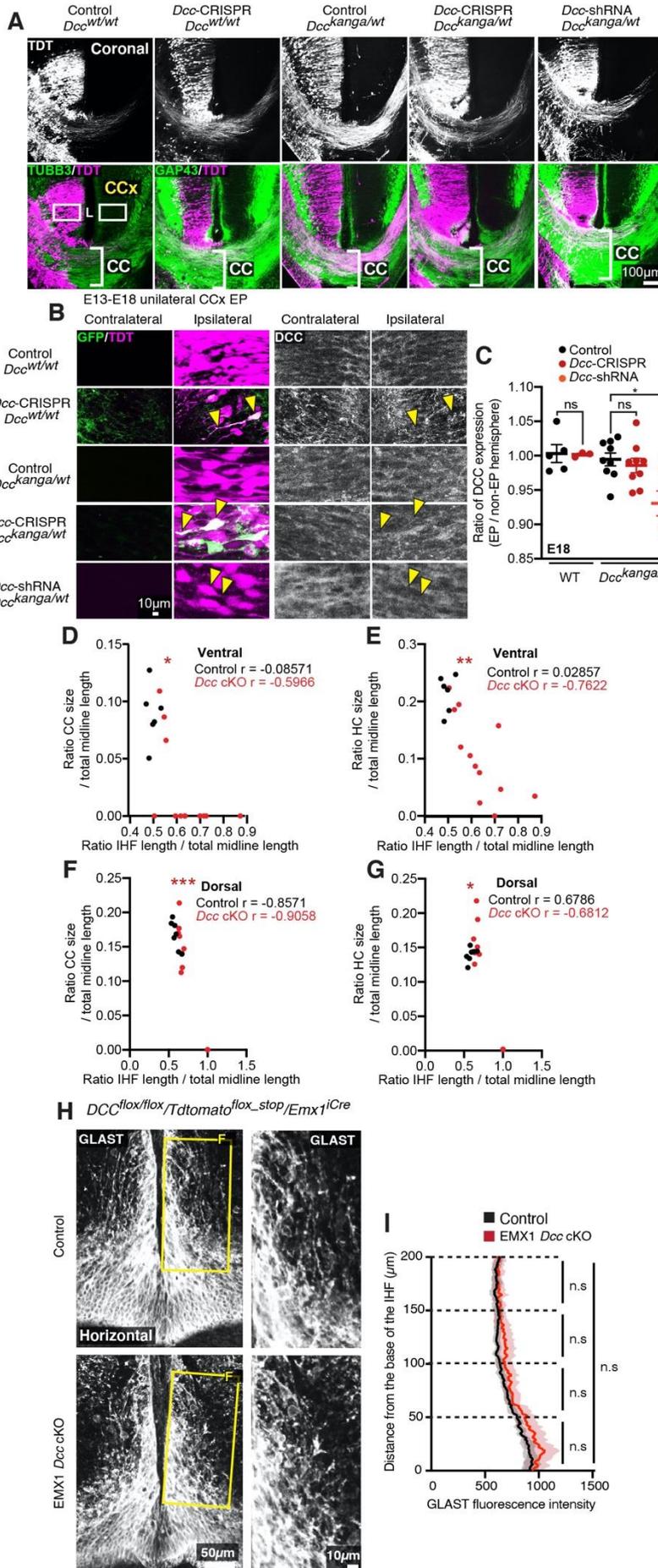


Figure 6-figure supplement 1: *Dcc* knockdown via targeted in utero electroporation does not cause CC abnormalities.

(A) TUBB3 (green) and TDT (white or magenta) in E18 *Dcc^{kanga}* mice electroporated with pCAG-TDTOMATO and either *Dcc*-CRISPR or *Dcc*-shRNA constructs into the CCx at E13. The CC is outlined with white brackets and white boxes indicate the location of panels represented in B.

(B) GFP (green), TDT (magenta) or DCC (white) in E18 *Dcc^{kanga}* mice electroporated with pCAG-TDTOMATO and either *Dcc*-CRISPR or *Dcc*-shRNA constructs into the CCx at E13. GFP indicates expression of the *Dcc*-CRISPR and yellow arrowheads indicate the

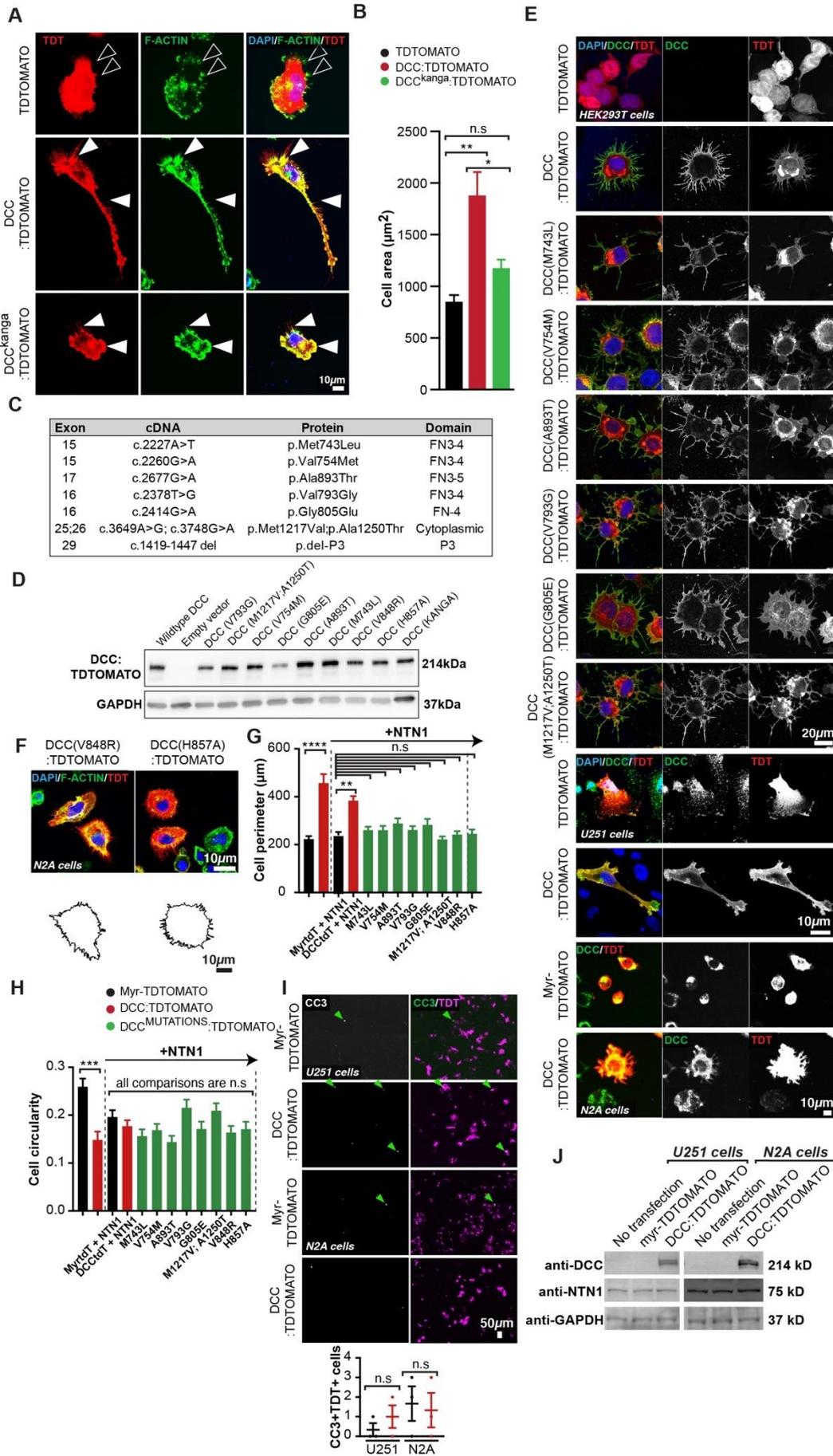
1592 location of select electroporated cells.

1593 (C) Quantification of the ratio of DCC expression between ipsilateral (electroporated;
1594 EP) and contralateral (non-electroporated) hemispheres shown in B.

1595 (D-G) Scatterplots of the relationship between CC length or HC length normalised to
1596 total telencephalic midline length and IHF length normalised to total telencephalic
1597 midline length for ventral and dorsal horizontal sections of P0 *Dcc* cKO mice.
1598 Nonparametric Spearman r correlations are shown.

1599 (H) Glial marker GLAST (white), in E15 *Dcc* cKO mice demonstrates the distribution
1600 of GLAST-positive MZG. Yellow boxes indicate region shown in insets, right and
1601 quantified in E.

1602 (I) Quantification of mean GLAST fluorescence within the telencephalic hinge from
1603 insets in D.



1605 **Figure 7-figure supplement 1: Mutant DCC receptors are expressed and**
1606 **trafficked normally but are unable to modulate cell shape**

1607 (A) Representative images of U251 glioblastoma cells immunolabelled for
1608 TDTOMATO (red), and F-actin (green) following transfection with plasmids encoding
1609 TDTOMATO, DCC:TDTOMATO, or DCC^{kanga}:TDTOMATO demonstrating
1610 predominant presence or absence of colocalised TDTOMATO with F-actin
1611 (arrowheads).

1612 (B) Quantification of average cell area from U251 cells represented in A.

1613 (C) Specific missense mutations were introduced into mouse pCAG-
1614 DCC:TDTOMATO and exon 29 was removed (del = deleted) to create the
1615 DCC^{kanga}:TDTOMATO construct.

1616 (D) COS-7 cells were transfected with pCAG-DCC:TDTOMATO constructs, including
1617 those carrying specific point mutations and the DCC^{kanga}:TDTOMATO construct.
1618 After 48 hours, cells were lysed, and a western blot was performed for mouse DCC
1619 and GADPH. Specific bands at 214kD and 37kD are shown.

1620 (E) HEK293T, N2A and U251 cells were transfected with pCAG-DCC:TDTOMATO
1621 constructs, including those carrying specific point mutations. After 24 hours, cells
1622 were fixed and immunohistochemistry was performed for the N-terminal of DCC
1623 without permeabilisation to detect membrane-inserted DCC (HEK293T) or for the C-
1624 terminal of DCC with permeabilisation (N2A and U251 cells).

1625 (F) Representative images of N2A cells immunolabelled for TDTOMATO (red), and
1626 F-actin (green) following transfection with plasmids encoding DCC:TDTOMATO
1627 carrying missense mutations and stimulated with recombinant mouse NTN1 protein
1628 with cell perimeter outlined below. The cell perimeter and cell circularity of these
1629 cells, and those represented in Figure 7B are quantified in (G) and (H) respectively.

1630 (I) Representative images of U251 and N2A cells immunolabelled for TDTOMATO
1631 (magenta), and cleaved-caspase3 (CC3; green) following transfection with plasmids
1632 encoding DCC:TDTOMATO and myr-TDTOMATO. Arrowheads indicate
1633 TDTOMATO-positive/CC3-positive cells, which are quantified below.

1634 (J) U251 and N2A cells were transfected with plasmids encoding myr-TDTOMATO
1635 and DCC:TDTOMATO or not transfected. After 20 hours, cells were lysed, and a
1636 western blot was performed for mouse DCC, NTN1 and GADPH. Specific bands at
1637 214kD, 75kD and 37kD are shown from n = 3 biological replicates.

1638 All graphs represent mean \pm SEM. Statistics by Kruskal-Wallis test for multiple
1639 comparisons: n.s = not significant with $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,
1640 **** $p < 0.0001$. See related Figure 6, Figure 7 and Supplementary File 1.

1641 All graphs represent mean \pm SEM. Statistics by Mann-Whitney test or unpaired t test:
1642 * $p < 0.05$, n.s = not significant with $p > 0.05$. See related Figure 7, Figure 8 and
1643 Supplementary File 1.

1644
1645 **Figure 1-source data 1:** Ratio of IHF length / total telencephalic midline length in
1646 *Dcc* and *Ntn1* mouse mutants

1647
1648 **Figure 3-source data 1:** Fluorescence intensity of GLAST and ratio of glial
1649 distribution / total midline length in *Dcc* mouse mutants

1650
1651 **Figure 4-source data 1:** Number and distribution of SOX9-positive MZG in *Dcc*
1652 mouse mutants

1653
1654 **Figure 5-source data 1:** Normalised fluorescence intensity of GFAP adjacent to the
1655 telencephalic midline in E17 *Dcc* and *Ntn1* mutant mice

1656
1657 **Figure 6-source data 1:** Measurements of IHF, CC and HC length and depth, DCC
1658 fluorescence and GLAST-positive/TDTOMATO-positive MZG cell bodies in *Dcc* cKO
1659 mice

1660
1661 **Figure 7-source data 1:** U251 or N2A cell area, perimeter and circularity following
1662 overexpression of DCC:TDTOMATO or myr-TDTOMATO

1663
1664 **Figure 8-source data 1:** N2A cell area following overexpression of
1665 DCC:TDTOMATO, DCC:TDTOMATO carrying a mutation or myr-TDTOMATO

1666
1667 **Figure 3-figure supplement 1-source data 1:** Fluorescence intensity of β -DYST, β -
1668 CAT, APC and N-CAD along the IHF surface in *Dcc*^{kanga} mice

1669 **Figure 4-figure supplement 1-source data 1:** Number of cells expressing EdU and
1670 Ki67, Cleaved-caspase 3 and SOX9 along the IHF surface in *Dcc*^{kanga} mice

1671

1672 **Figure 5-figure supplement 1-source data 1:** Fluorescence intensity
1673 measurements for GFAP, *Fgf8* and *Mmp-2* mRNA, and quantification of NFI-
1674 positive/GLAST-positive cell bodies in *Dcc^{kanga}* mice

1675

1676 **Figure 6-figure supplement 1-source data 1:** Quantification of the ratio of DCC
1677 expression between hemispheres, measurements of IHF length, CC length and HC
1678 length, and GLAST fluorescence intensity along the IHF surface in *Dcc cKO* mice

1679

1680 **Figure 7-figure supplement 1-source data 1:** U251 cell area, N2A cell perimeter
1681 and circularity and cleaved-caspase3 expression following overexpression of
1682 DCC:TDTOMATO, DCC:TDTOMATO carrying a mutation, Myr-TDTOMATO, or
1683 TDTOMATO alone.

1684

1685 **Supplementary file 1: Statistics**

1686 Statistics related to quantified data in figures 1-8 and figure 1-7 supplements. CA =
1687 cell area, CP = cell perimeter, CC3 = cleaved-caspase 3, DCCK = DCCK^{kanga}, E =
1688 embryonic day, EP = electroporated, exp = experimental, FI = fluorescence intensity,
1689 IGG = indusium griseum glia, MZG = midline zipper glia, P = postnatal day, ROI =
1690 region of interest, TDT = TDTOMATO, vs. = versus, wt = wildtype.

1691

1692 **Supplementary file 2: Key resources table**

1693 Resources used to generate the data contained within figures 1-8 and figure 1-7
1694 supplements. See materials and methods for further details.