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Non-newly generated, “immature” neurons in the sheep brain are not restricted to cerebral cortex

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**Non-newly generated, "immature" neurons
in the sheep brain are not restricted to cerebral cortex**

Abbreviated title: **Immature neurons in the sheep brain**

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38 **Abstract**

39

40 A newly proposed form of brain structural plasticity consists of non-newly generated, "immature"
41 neurons of the adult cerebral cortex. Similar to newly generated neurons, these cells express the
42 cytoskeletal protein Doublecortin (DCX), yet they are generated prenatally, then remaining in a
43 state of immaturity for long periods. In rodents, the immature neurons are restricted to the
44 paleocortex, whereas in other mammals are found also in neocortex. Here, we analyzed the DCX-
45 expressing cells in the whole sheep brain of both sexes, to search for an indicator of structural
46 plasticity at a cellular level in a relatively large-brained, long-living mammal. Brains from adult and
47 newborn sheep (injected with BrdU and analyzed at different survival times) were processed for
48 DCX, cell proliferation markers (Ki-67, BrdU), pallial/subpallial developmental origin (*Tbr1*, *Sp8*),
49 and neuronal/glial antigens for phenotype characterization. We found immature-like neurons in the
50 whole sheep cortex and in large populations of DCX-expressing cells within the external capsule
51 and the surrounding grey matter (claustrum and amygdala). BrdU and Ki-67 detection at neonatal
52 and adult ages showed that all these DCX+ cells were generated during embryogenesis, not after
53 birth. These results show that the adult sheep, unlike rodents, is largely endowed with non-newly
54 generated neurons retaining immature features, suggesting that such kind of plasticity might be
55 particularly important in large brained, long living mammals.

56 **Significance statement**

57 Brain plasticity is important in adaptation and brain repair. Structural changes span from synaptic
58 plasticity to adult neurogenesis, the latter being highly reduced in large-brained, long-living
59 mammals (e.g., humans). The cerebral cortex contains "immature" neurons, which are generated
60 prenatally then remaining in an undifferentiated state for long periods, being detectable with
61 markers of immaturity. We studied the distribution and developmental origin of these cells in the
62 whole brain of sheep, namely, relatively large-brained, long-living mammals. In addition to the
63 expected cortical location, we also found populations of non-newly generated neurons in several
64 subcortical regions (external capsule, claustrum, amygdala). These results suggests that non-
65 neurogenic, parenchymal structural plasticity might be more important in large mammals with
66 respect to adult neurogenesis.

67 **Introduction**

68 The mammalian central nervous system (CNS) is build up mostly of non-renewable (perennial)
69 neurons whose cell processes are connected physically and functionally in a largely invariant way.
70 Though anatomical invariability is a necessary requisite to assure stability of connections in the
71 neural circuits (Frotscher, 1992), exceptions do exist in the form of cellular modifications referred
72 to as "structural plasticity", affecting the brain anatomy at different levels and degrees (Bonfanti,
73 2006; Theodosis et al., 2008). These exceptions span from formation/elimination of synapses in pre-
74 existing neurons (synaptic plasticity; Bonfanti and Theodosis, 2009; Bailey et al., 2015) to
75 addition/replacement of new neurons (adult neurogenesis; Bonfanti and Peretto, 2011; Aimone et
76 al., 2014). The occurrence, amount, type and location of neural structural plasticity, as well as its
77 reparative capacity, greatly vary in the animal world (Bonfanti, 2011; Grandel and Brand, 2013)
78 and, to a lesser extent, among mammals (Feliciano et al., 2015; Lipp and Bonfanti, 2016). The
79 cytoskeletal protein doublecortin (DCX) is an excellent marker for cells that retain high potential
80 for structural plasticity in the CNS (Gleeson et al., 1999; Nacher et al., 2001). Due to its heavy
81 expression in newly generated neuroblasts and during the early phases of their
82 migration/differentiation, DCX is commonly used as a marker for adult neurogenesis (Brown et al.,
83 2003). Nevertheless, it is now clear that at least a type of neurons located in the layer II of the adult
84 mammalian cerebral cortex, which are not newly generated ("immature neurons", Gomez-Climent
85 et al., 2008), express DCX during adulthood (for review, see Bonfanti and Nacher, 2012; Nacher
86 and Bonfanti, 2015). Current data indicate that the occurrence and distribution of DCX+ cells can
87 substantially vary in brain regions of different mammals (Feliciano et al., 2015; Lipp and Bonfanti,
88 2016), thus suggesting species-specific heterogeneity in the capability to undergo structural
89 plasticity, both neurogenic and non-neurogenic. For instance, the rate of adult neurogenesis
90 decreases in mammals with extended life expectance (e.g., humans, dolphins and sheep; Sanai et al.,
91 2011; Brus et al., 2013a; Parolisi et al., 2015,2017) if compared with the relatively short-living

92 laboratory rodents (reviewed in Paredes et al., 2015; Lipp and Bonfanti, 2016). By contrast, the
93 occurrence of cortical layer II immature neurons is higher in rabbits, guinea pigs and cats with
94 respect to rats and mice, extending into neocortical regions in the former and being restricted to
95 paleocortex in the latter (Luzzati et al., 2009; Cai et al., 2009; Xiong et al., 2008). Here, the
96 occurrence of DCX+ cells was investigated in sheep (*Ovis aries*) with the idea of analysing the
97 distribution of this indicator of structural plasticity in a relatively long-living mammal endowed
98 with a large-sized, gyrencephalic brain. Sheep possess a brain as large as a macaque monkey and
99 have a similar life span (10/30 years in wild/captivity); also, experimental procedures such as BrdU
100 injection for subsequent immunocytochemical detection of newly generated cells in the brain tissue
101 can be performed in these animals. Neonatal and adult animals treated with BrdU (injected in
102 pregnant ewes in the case of neonates) were studied in order to assess the time of genesis (prenatal
103 vs. postnatal) of the DCX+ cells. We show that in the adult sheep brain, in addition to cortical
104 immature neurons, different populations of non-newly generated DCX+ cells are consistently
105 present in the external capsule and adjacent regions. Interestingly enough, quantification of these
106 latter cells in neonatal, prepuberal and adult animals showed they are not depleted through ages.

107

108

109

110 **Materials and Methods**

111

112 **Animals, BrdU injections, tissue preparation**

113 Neonatal, prepuberal and adult animals (Ile de France) were raised at the INRA research center
114 (Nouzilly; Indre et Loire, France). Experiments were conducted on 9 adult (females, 2 year old), 3
115 prepuberal (males, 4 month old), and 7 neonatal animals (4 males, 3 females, 1 week old; see Table

116 1). Adult ewes were housed in an individual pen (2x1 m) and received four intravenous injections
117 of bromodeoxyuridine (BrdU) during pregnancy (1 injection/day, 20 mg/Kg in 0.9% saline; Sigma-
118 Aldrich, France), a thymidine analogue incorporated into the DNA during the S-phase of the mitotic
119 division. Two days after lambing, ewes were anesthetized with thiopental and decapitated by a
120 licensed butcher in an official slaughterhouse (ethical permissions reported in Brus et al., 2013b).
121 Three different survival times were analyzed in these adult animals: 1, 2 and 4 months (see Brus et
122 al., 2013b). Since all the ewes were pregnant, the intravenous injections of BrdU could allow the
123 molecule to pass to the fetuses and thus being incorporated in their brain. All the lambs used in this
124 study were collected from mothers being injected 3 months before parturition (i.e. at 2-month
125 gestational days). Brains were perfused through both carotid arteries with 2L of 1% sodium nitrite
126 in phosphate buffer saline, followed by 4 L of ice-cold 4% paraformaldehyde solution in 0,1M
127 phosphate buffer at pH 7,4. The brains were then dissected out, cut into blocks and post-fixed in the
128 same fixative for 48h. The tissues were then stored in 30% sucrose. Each hemisphere has been cut
129 into 4 coronal slices (about 1,5 cm thick), embedded in OCT (optimum cutting temperature, Bio-
130 Optica), frozen in isopentane, and stored at -80°C. Cryostat coronal sections (40 µm tick) were cut
131 to be employed in free-floating immunohistochemistry and immunofluorescence procedures. We
132 then obtained the outlines of four levels of interest, representing the whole sheep brain (L1-L4), by
133 combining the analysis of our cryostat sections and photographs from the atlas of Brain
134 Biodiversity Bank of Michigan State University (Fig. 1).

135

136

137 **3.2 Immunohistochemistry**

138 Immunohistochemical reactions were performed on free-floating sections, when necessary, antigen
139 retrieval was performed using citric acid at 90°C for 5-10 minutes. The section were incubated in
140 blocking buffer (2% normal serum, 0,5-1% Triton X-100 in 0,01M PBS, ph 7.4) for 2h at room

141 temperature, then incubated for 24-48 h at 4 °C in a solution of 0.01 M PBS, pH 7.4, containing 0,5-
142 1% Triton X-100, 2% normal serum and the primary antibodies (see Table 2). Sections were then
143 incubated with appropriate solutions of secondary antibody: Alexa-488 conjugated goat anti mouse
144 (1:400, Molecular Probes, Eugene, OR), Alexa-488 conjugated goat anti rabbit (1:400, Molecular
145 Probes, Eugene, OR), Alexa-488 conjugated donkey anti rat (1:400 Jackson ImmunoResearch,
146 West Grove, PA), Alexa-555 conjugated goat anti mouse (1:800, Molecular Probes, Eugene, OR),
147 Alexa-555 conjugated goat anti rabbit (1:800, Molecular Probes, Eugene, OR), Alexa-555
148 conjugated goat anti guinea pig (1:800, Molecular Probes, Eugene; OR), cyanine 3 (cy3) conjugated
149 goat anti mouse (1:800, Jackson ImmunoResearch, West Grove, PA), cyanine 3 (cy3) conjugated
150 goat anti rabbit (1:800, Jackson ImmunoResearch, West Grove, PA), cyanine 3 (cy3) conjugated
151 donkey anti goat (1:800, Jackson ImmunoResearch, West Grove, PA), Alexa-647 conjugated donkey
152 anti mouse (1:800, Jackson ImmunoResearch, West Grove, PA), Alexa-647 conjugated donkey anti
153 rabbit, for 3h at RT. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, KPL,
154 Gaithersburg, MD), coverslipped with MOWIOL 4-88 (Calbiochem, Lajolla, CA) and examined.
155 For 3,3'-diaminobenzidine (DAB) immunohistochemistry sections were incubated in a solution of
156 0,3% H₂O₂ in 0,01 M PBS, pH 7.4 for 15 minutes to inhibit the endogenous peroxidase, before the
157 incubation with blocking buffer. Following primary antibody incubation sections were incubated
158 with goat anti rabbit IgG biotinylated secondary antibody (1:350, Vector Laboratories, Burlingame,
159 CA) or horse anti goat IgG biotinylated secondary antibody (1: 250, Vector Laboratories,
160 Burlingame, CA) for 2h at RT. Sections were washed and incubated in avidin-biotin-peroxidase
161 complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 2h at RT. The reaction
162 was developed with DAB Peroxidase Substrate Kit, 3,3'-diaminobenzidine (Vector laboratories,
163 Burlingame, CA) for 3-5 minutes. Sections were mounted on TESPA (3-aminopropyl-
164 triethoxysilan) treated slides and then counterstained with 1% cresyl violet acetate solution (1-2
165 minutes exposure) and coverslipped with Neo-Mont 109016 (Merck, Darmstadt, Germany).

166

167 **3.3 Quantitative analyses**

168 Cell counting was performed using NeuroLucida software (MicroBrightfield, Colchester, VT).

169 *Diameters of DCX+ objects* (see below, in Results) *in the external capsule and pericapsular*
170 *regions*: the average object diameter (orthogonal to main axis) has been measured for all the
171 clusters and groups of scattered cells in the external capsule, claustrum and amygdala of 3 adult
172 animals using the straight line tool of ImageJ program after a proper calibration, then reporting the
173 minimum and maximum length to obtain a size range.

174 *Density of DCX+ objects in the external capsule at neonatal and adult ages*: the number of DCX+
175 cell clusters and groups has been counted in the whole extension of the external capsule in 3 adult
176 and 3 newborn animals, using a serial step of 12 cryostat sections (480 μm ; 11 sections for the adult
177 and 10 for the newborn). The density was calculated as the total number of objects/area (mm^2). A 3-
178 D reconstruction aimed at further characterize the DCX+ clusters in the adult has been made in the
179 posterior part of the external capsule (24 serial cryostat sections, 40 μm thick, corresponding to 0,96
180 mm of white matter tissue).

181 *Cell density in the amygdala and claustrum at neonatal and adult ages*: the total number of DCX+
182 cells present within the two pericapsular regions have been counted in 3 adult and 3 newborn
183 animals (three slices corresponding to the anterior, middle and posterior part of each anatomical
184 structure have been considered). The density was calculated as the total number of cells/area (mm^2).

185 *Linear density of DCX+ neurons in the cerebral cortex at neonatal and adult ages*: the DCX+ cells
186 present in the cortical layer II have been counted within two brain levels (L2 and L3) and three
187 regions (the cingulate cortex and the medial margin of the suprasylvian gyrus in the neocortex; the
188 piriform cortex in the paleocortex; see Fig. 6), using three cryostat sections/level, in 3 adult and 3
189 newborn animals. The linear density was calculated as the total number of cells/the cortical tract
190 length (mm).

191 *Cell soma diameters of DCX+ cells:* the average cell soma diameter (orthogonal to main axis) has
192 been measured for 200 cells in the cortex, claustrum and amygdala of 3 adult animals using the
193 *straight line* tool of ImageJ program after a proper calibration, then reporting the minimum and
194 maximum length to obtain a size range.

195 *DCX+/BrdU+ double staining:* the percentage of double-stained cells has been calculated after
196 analyzing 200 cells in the cortex, claustrum, external capsule and amygdala of 3 newborn (number
197 of DCX+/BrdU+ cell out of DCX+, single-stained cells) and 3 adult animals (for each survival
198 time: 1, 2 and 4 months).

199 *DCX+/NeuN+ double staining:* the percentage of double-stained cells has been calculated after
200 analyzing 200 cells in the cortex, claustrum and amygdala of 3 adult animals (number of
201 DCX+/NeuN+ cells out of DCX+, single-stained cells).

202 *DCX+/Tbr1+ and DCX+/Sp8+ double staining:* the percentage of double-stained cells has been
203 calculated after analyzing 200 cells in the cortex, external capsule, claustrum and amygdala of 3
204 newborn animals (number of DCX+/Tbr1+ or DCX+/Sp8+ cells out of DCX+, single-stained
205 cells).

206 *Statistical Analysis:* all graphics and relative statistical analysis have been made using GraphPad
207 Prism 5 Software (La Jolla, CA, USA), and included unpaired (two-tailed) Student's t test
208 (comparing only two groups), and two-way ANOVAs. $p < 0.05$ was considered as statistically
209 significant. Data are expressed as averages \pm standard deviation (SD).

210

211 **3.4 Image acquisition and processing**

212 Images from immunofluorescence specimens were collected with Leica TCS SP5 (Leica
213 Microsystems, Wetzlar, Germany) confocal microscope. Images from DAB immunohistochemistry
214 were collected with eclipse 80i Nikon microscope (Nikon, Melville, NY) connected to a color CCD
215 Camera. Images were processed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA) and

216 ImageJ version 1.50b (Wayne Rasband, Research Services Branch, National Institute of Mental
217 Health, Bethesda, Maryland, USA). Only general adjustments to color, contrast, and brightness
218 were made. The 3D reconstruction in the external capsule was performed using NeuroLucida
219 software (MicroBrightfield, Colchester, VT) by aligning 24 consecutive coronal sections starting
220 from the onset of the thalamus. The sections were previously immunoreacted for DCX, using DAB
221 peroxidase staining and counterstained with 1% cresyl violet acetate solution.

222

223

224 **Results**

225

226 **Distribution of DCX+ cells in the adult sheep brain**

227 After systematic screening of the whole adult sheep brain several populations of DCX+ cells were
228 detected at different locations (Fig. 2): i) newly generated neuroblasts/neuronal-like cells within the
229 SVZ and hippocampal neural stem cell niches, ii) neuronal-like cells in the superficial layers of the
230 cerebral cortex, and iii) groups of neuroblasts/neuronal-like cells in the external capsule and
231 surrounding grey matter (claustrum and amygdaloid nuclei). No DCX+ cells were detected in the
232 striatum/putamen. In addition to their different location, these DCX+ cells appeared to vary in their
233 morphology and spatial organization (Fig. 2B,C). Cells in the neurogenic zones typically appeared
234 as previously described (Brus et al., 2010; 2013b): single elongated, bipolar neuroblasts in the SVZ
235 adjacent to the lateral ventricle wall and at the lower aspect of the hippocampal dentate gyrus (Fig.
236 2D); these neurogenic regions were used as an internal control for detection of local cell division
237 markers (Ki-67 antigen) and newly generated neurons identifiable through injection of exogenous
238 markers (BrdU) and subsequent detection at different survival times.

239

240 *DCX+ cells in the cerebral cortex*

241 As previously described in some cortical areas of other mammals (reviewed in Bonfanti and
242 Nacher, 2012), DCX+ neuronal-like cells were detected in the cerebral cortex of the adult sheep
243 (Fig. 3). These cells were present in the superficial layers of most paleo- and neo-cortex (Figs. 3A
244 and 3B). They were further characterized in order to use them as a population of DCX+ cortical
245 neurons which has been already described in other species, here studied in sheep. In the cortical
246 cytoarchitecture the DCX+ neuronal-like cells were localized in the upper part of layer II (at the
247 limit with layer I), being present along the entire antero-posterior and dorso-ventral axis of the
248 brain. They appeared to be more abundant in the piriform cortex (paleocortex) with respect to
249 neocortex (Fig. 2B; see below for quantitative analysis). Two main morphological types falling into
250 two cell body size categories were identified: small cells, diameter 4-7 μm (type 1 cells), and large
251 cells, diameter 9-12 μm (type 2 cells; Fig. 3C,D). The large-sized cells frequently appeared as
252 pyramidal-like neurons (similar to the "principal cells" previously described in rats by Gomez-
253 Climent et al., 2008), whereas the small cells mostly showed a simpler, bipolar morphology (Fig.
254 3C,D). The principal cell type can be further split into two main patterns linked to soma shape and
255 dendritic complexity: those with oval-shaped cell body and poorly ramified apical dendrites (here
256 referred to as type 2a) and others with more developed apical dendrite arborization and basal
257 dendrites (here referred to as type 2b; Fig. 3C,D). Type 2 cells were far less abundant with respect
258 to type 1 cells (around 7%; Fig. 3D).

259

260 *DCX+ cells in the external capsule and surrounding regions*

261 Additional DCX+ cell populations were detectable in the external capsule and surrounding regions
262 (claustrum and amygdala). Most of these cells were grouped into discrete bulks to form either
263 tightly-packed cell clusters (mainly in the white matter; Fig. 2E), or groups of scattered cells (more
264 frequently observed in grey matter; Fig. 2G). Due to their heterogeneity, and in order to perform a
265 quantitative analysis, they were considered as "DCX+ objects", divided into compact cell clusters

266 (within the external capsule) and groups of scattered cells (in the surrounding regions: amygdala
267 and claustrum).

268 *External capsule.* Most DCX⁺ cells in capsular white matter appeared as bipolar, elongated
269 elements, often endowed with very long cell processes, assembled to form tightly-packed cell
270 clusters (Fig. 4C). These clusters showed remarkable diameter variability (between 50 and 380 μm).

271 When observed in single coronal sections, some of them were reminiscent of the thick chains of
272 neuroblasts described in the SVZ of rodents (Lois et al., 1996) and in the surrounding regions of
273 rabbits (Luzzati et al., 2003; Ponti et al., 2006). Hence, a 3D reconstruction was performed through
274 an anterior-posterior portion of the external capsule (Fig. 4B; see Methods) in order to assess their
275 relationship with the SVZ neurogenic site. The analysis revealed that all clusters appear as discrete
276 bulks of cells not forming long streams and never contacting the SVZ neurogenic site. In order to
277 investigate the relationship with surrounding cells (e.g., the occurrence of specific astrocytic
278 arrangements), immunocytochemistry for glial fibrillary astrocytic protein (GFAP) was performed.
279 GFAP staining did not revealed any special density or niche-like organization adjacent to the DCX⁺
280 cells (Fig. 4D); yet, when the DCX⁺ cells were organized to form tightly-packed clusters they
281 occupied glial-empty spaces, the astrocytes being segregated outside them (Fig. 4D). Quantification
282 of DCX⁺ cell clusters (see below and Table 3) revealed that virtually all of them were contained
283 within the external capsule, mostly residing in its posterior part (Fig. 4B). This analysis further
284 excluded any direct contact between the clusters and the SVZ neurogenic site. Groups of less
285 tightly-packed cells and scattered/isolated cells were also observed (Fig. 4C).

286 *Amygdala and claustrum.* Compact cell clusters were rare in the peri-capsular grey matter, whereas
287 populations of scattered DCX⁺ cells were prevalent (Fig. 5). These cell groups were larger in the
288 amygdala (diameter ranging between 125 and 385 μm) than in claustrum (40 to 200 μm). Within
289 both regions, large multipolar DCX⁺ neurons endowed with long, dendritic-like processes were
290 mixed with smaller, bipolar cells (Fig. 5B,C). Bipolar cells represented by large the most frequent
291 cell morphology (around 98-99%; Fig. 5D). Three main cell morphologies were present in the

292 amygdala (Fig. 5B): type 1 cells (small, bipolar; soma diameter 3-6 μm), type 2 cells (medium-
293 sized, similar to type 2a cells in cortex; soma diameter 8-9 μm), and type 3 cells (large, multipolar;
294 soma diameter 9-11 μm). Two main cell types were detectable in the claustrum (Fig. 5C): type 1
295 cells (small, unipolar or bipolar; soma diameter 3-6 μm), type 2 cells (soma diameter 7-9 μm).

296

297 **DCX+ cell populations at different ages**

298 The same types of analyses performed on the DCX+ cells in the cortex, capsular and pericapsular
299 regions were carried out on early postnatal brains (7 days after birth). Under a qualitative profile, by
300 comparing the occurrence and distribution of DCX+ cell populations at different ages, the only
301 substantial difference concerned the presence of cell clusters within the putamen of the newborn
302 and within the corpus callosum of neonatal and prepuberal animals (not shown), both cell
303 populations then disappearing in adults (article in preparation). All other DCX+ cell populations,
304 including cortical immature neurons, neuroblasts of the neurogenic sites, and DCX+ cells in
305 capsular/pericapsular regions were present at both ages. Yet, quantitative evaluations carried out in
306 neonates and adults revealed differences among these regions (Fig. 6). When compared to
307 newborns, the cerebral cortex of adult animals showed an evident and generalized decrease in the
308 amount of DCX+ immature neurons (Fig. 6A, top). To quantify this reduction we calculated the
309 linear density of layer II DCX+ cells in three cortical segments, including both neocortex and
310 paleocortex of neonatal and adult sheep (see Methods and Fig. 6A, bottom). In all analyzed regions
311 both the number and density of DCX+ cells underwent approximately a four-fold reduction with
312 age (see Table 3 for statistical comparisons). Such trend, suggesting an age-related reduction in
313 cortical plasticity of the layer II (as previously shown in several species; Abrous et al., 1997; Varea
314 et al., 2009), parallels the remarkable drop described for the DCX+ cell populations in the
315 neurogenic sites of different mammals (Sanai et al., 2011; Lipp and Bonfanti, 2016) which is also
316 strikingly evident in both SVZ and hippocampus of sheep (Fig. 6B).

317 Interestingly enough, in contrast with their neocortical counterparts, both the number and the
318 density of DCX+ cell populations in the capsular and pericapsular regions were substantially stable
319 at both ages (Fig. 6C and Table 3 for statistics). The slight reduction in the density of DCX+ cell
320 clusters detectable in the external capsule is related to the relative increase in the area of this region
321 with respect to others (increasing volume of the capsule itself; see Table 3). Two way ANOVA
322 analyses confirmed the presence of a significant interaction between brain region and age for both
323 number ($F=136,551$; $p<0.0001$) and density of DCX+ cells/objects ($F=84,258$; $p<0.0001$). Pairwise
324 comparisons clearly showed that subcortical regions had a similar age related trend that differed
325 from that of both paleo and neocortex (Table 3). As to the topographical localization of the DCX+
326 objects within each brain region, these structures were located more posteriorly in the adult external
327 capsule, whereas in the newborn they were distributed homogeneously along the entire antero-
328 posterior axis. In claustrum and amygdala the distribution was generally homogeneous (in the latter,
329 mainly located in the basolateral nucleus).

330

331 **Cell proliferation analysis**

332 The heavy occurrence of DCX+ cells in the cerebral cortex, external capsule and surrounding
333 regions of the sheep brain opens the question whether they are newly generated. Analysis with Ki-
334 67 antigen and BrdU in adults consistently revealed immunopositive nuclei in both SVZ and
335 hippocampal neurogenic sites, here used as internal controls for detection of cell proliferation
336 markers and neurogenesis (see Fig. 2). By contrast, no Ki-67/DCX colocalization was detectable in
337 all cortical, capsular/pericapsular regions analyzed (Fig. 7A), as well as no BrdU staining were
338 found in association with parenchymal DCX+ cells in any of the adult animals injected with the
339 exogenous cell proliferation marker and subsequently analysed at 30, 60, 120 days survival (Fig.
340 7A). The results obtained by joining local cell proliferation marker and BrdU pulse labelling

341 analyses strongly indicate that the external capsule/pericapsular DCX+ cells are not dividing at any
342 of the adult ages considered, thus excluding the occurrence of parenchymal neurogenesis.

343 To confirm that these cell populations were generated earlier, during embryogenesis, DCX/BrdU
344 double staining was performed in lambs born from mothers injected with BrdU 3 months before
345 parturition (Fig. 7B). Numerous BrdU+ nuclei were consistently present in all areas analysed. In the
346 external capsule of neonates, some of them were detectable in DCX+ cells of the tightly-packed
347 clusters as well as in isolated cells (Fig. 7B). Some BrdU+ cells were DCX-negative, likely
348 corresponding to post-mitotic (mature) neurons which had been generated during embryogenesis
349 and having already lost their DCX staining (see below). DCX+ cells not immunoreactive for BrdU
350 were also present, indicating immature cells generated at previous or later developmental stages.
351 After analysis with Ki-67 antigen in neonates, some scattered immunopositive nuclei were
352 detectable at different locations of the brain parenchyma, yet never involving double-staining with
353 DCX+ cells (Fig. 7B), thus indicating that these latter are no more proliferating after birth. A similar
354 pattern was observed in the cortex where no Ki-67+/DCX+ or BrdU/DCX+ cells were detectable in
355 the adult, whereas BrdU+/DCX+ cells were systematically detected in cortical layer II of neonates;
356 these cells were far more abundant in the neocortex with respect to the paleocortex (see pie charts in
357 Fig. 7B). Similarly to what observed in capsular/pericapsular regions, some Ki-67+ nuclei were
358 present in the cortex of neonates but never in double staining with DCX.

359

360 **Cell maturity/immaturity and pallial/subpallial origin markers**

361 In order to get some insight concerning the degree of maturation of the DCX+ cell populations
362 described here, we used markers commonly employed to assess their neuronal maturational stage.
363 We analysed NeuN, an RNA-binding protein expressed by most postmitotic neurons which start
364 differentiation (Mullen et al., 1992; Fig. 8A). Only a small percentage of the DCX+ cells (around
365 10%) co-expressed NeuN in different regions (11,7% in cortical layer II; 9,1% in claustrum; 8,3%

366 in amygdala; Fig. 8B). In the cortex, all the DCX+/NeuN+ neurons fell in the type 2 cell
367 morphology with ramified dendrites. In the external capsule NeuN was not detectable within the
368 tightly-packed cell clusters, whereas some isolated cells detached from the clusters were double-
369 stained (Fig. 8A). This gives support to the hypothesis that larger DCX+ cells in layer II are slightly
370 more mature than small cells, showing increases in NeuN expression as described during neuronal
371 differentiation in the adult dentate gyrus (Kempermann et al., 2004; Marques-Mari et al., 2007).
372 Similarly, another marker of mature neuronal cells (HuC/D RNA-binding protein; Barami et al.,
373 1995) was detected only in some of the cortical DCX+ neurons (again, mostly large, type 2 cells;
374 Fig. 8C). In parallel, subpopulations of DCX+ cells in all regions investigated were immunoreactive
375 for PSA-NCAM, a marker of immaturity expressed by cells retaining plasticity (Bonfanti, 2006).
376 Unlike newly generated neuroblasts of the classic neurogenic sites (SVZ and dentate gyrus) which
377 are mostly PSA-NCAM immunoreactive, in cortex, amygdala, claustrum and external capsule the
378 staining was detectable only in some DCX+ cells, being restricted to parts of their membrane (Fig.
379 8D). A similar pattern was observed with the A3 subunit of the cyclic nucleotide-gated ion channel
380 (CNGA3; Fig. 8E), which has been previously shown in immature cortical neurons (Varea et al.,
381 2011) and is considered involved in brain plasticity (Michalakis et al., 2011). The results obtained
382 with the above mentioned markers were substantially similar in all regions and ages considered. On
383 the whole, many DCX+ cells (of both types) also expressed markers of immaturity whereas only
384 small subpopulations (NeuN, mainly type 2 cells) or a few of them (HUC/D) expressed markers of
385 differentiation/maturity (summarized in Fig. 8F).

386 Once assessed the prenatal origin of the parenchymal DCX+ cells, the embryologic divisions
387 (neural progenitor domains) of their origin were investigated by employing two markers of pallial
388 (T-box transcription factor, *Tbr1*) and subpallial origin (zinc-finger protein, *Sp8*; experiments
389 carried out on neonates). The presence of these two proteins was analyzed in the various areas
390 investigated. As previously reported in other mammals, *Tbr1* was present in pallial derivatives such
391 as the hippocampus, claustrum, amygdala, and piriform cortex, being frequently associated with the

392 DCX+ neurons (Fig. 9). In the neocortex, *Tbr1* was strongly expressed in deeper layers with respect
393 to upper layers (where it was mainly found in type 1 cells of the layer II; Fig. 9A). Interestingly, in
394 cortical upper layers this transcription factor is downregulated during neuronal maturation, at least
395 in mice (Toma et al. 2014). Only rare cells were positive for *Sp8* in these two regions (2,2% in
396 cortex and 1,4% in claustrum). By contrast, the situation was more heterogeneous in the external
397 capsule and amygdala: two intermixed but distinct cell populations were immunopositive for each
398 one of the two markers, with a prevalence of *Tbr1*+ cells (Fig. 9B). These results strongly support
399 the view that the DCX+ immature cells in subcortical regions are generated from both subpallial
400 and pallial regions (about 25% and 75%, respectively) of the embryonic SVZ.

401

402

403 Discussion

404

405 The cytoskeletal protein DCX is associated with neuronal maturation and cell shape global
406 remodeling, thus being involved in structural plasticity (Nacher et al., 2001; Brown et al., 2003).
407 For decades, much attention has been drawn on adult neurogenesis as a striking process of plasticity
408 involving the production of new neurons which impact on learning and memory, also opening
409 possibilities for brain repair (Martino et al., 2011; Peretto and Bonfanti, 2014; Berninger and
410 Jessberger, 2016). In mammals, the functional integration of newborn neurons is highly restricted to
411 olfactory bulb and hippocampus (Bonfanti and Peretto, 2011), their stem cell niches being less
412 active in humans than in rodents (Sanai et al., 2011; Lipp and Bonfanti, 2016). An emerging form of
413 plasticity consists of cells retaining features of immaturity through adulthood, including the
414 persistent expression of DCX though they are not generated *de novo* postnatally ("immature
415 neurons", Gomez-Climent et al., 2008). Originally, these cells were described in the paleocortex of
416 rodents (Seki and Arai, 1991; Bonfanti et al., 1992). Their distribution and role remain largely

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Comment citer ce document :

Piumatti, M., Palazzo, O., La Rosa, C., Crociara, P., Parolisi, R., Luzzati, F., Lévy, F.,
Bonfanti, L. (2018). Non-newly generated, "immature" neurons in the sheep brain are not restricted
to cerebral cortex. *Journal of Neuroscience*, 1-50. , DOI : 10.1523/JNEUROSCI.1781-17.2017

417 object of investigation, systematic studies being scarce (Bonfanti and Nacher, 2012; König et al.,
418 2016). In some mammals, similar cells are also present in neocortex (Xiong et al., 2008; Cai et al.,
419 2009; Luzzati et al., 2009; Zhang et al., 2009), leading to speculate that non-neurogenic structural
420 plasticity might be prominent in non-rodent species (Bonfanti, 2016 and present work).

421 We screened the occurrence, location, distribution and developmental origin of DCX+ cells as an
422 indicator of non-neurogenic plasticity in sheep: long-living mammals endowed with relatively
423 large-sized, gyrencephalic brain. By using markers of cell division (Ki-67) and pulse labelling of
424 BrdU, we revealed the presence of abundant DCX+ cell populations born prenatally and not
425 generated after birth. These cells were not restricted to cerebral cortex, also occurring in white and
426 grey matter of pallial subcortical regions: external capsule, claustrum, amygdala. In contrast with
427 the substantial decrease in number of DCX+ cortical neurons at increasing ages (Abrous et al.,
428 1997; Xiong et al., 2008; Cai et al., 2009; Varea et al., 2009, here confirmed in sheep), the
429 subcortical DCX+ cells appear steadily maintained through time, at least in young adults (Figs. 6
430 and 10). Groups of DCX+ cells were previously found close to the external capsule in rabbits
431 (Luzzati et al., 2003) and in the amygdala of non-human primates (Zhang et al., 2009; de Campo et
432 al., 2012). A small portion of them were considered as newly generated in the amygdala of rabbits
433 (Luzzati et al., 2003), mouse (Jhaveri et al., 2017) and primates (Bernier et al., 2002). Our
434 experiments in the sheep excluded the occurrence of adult newborn neurons in any of the
435 parenchymal regions containing DCX+ cells, thus revealing species-specific heterogeneity in their
436 regional distribution.

437 As to the maturational stage of the cells, many of them expressed the markers of
438 immaturity/plasticity PSA-NCAM and CNGA3, in addition to DCX. Also the *Tbr1* expression in
439 DCX+ cortical cells (mainly of type 1) in layer II (wherein the transcription factor is usually
440 downregulated with maturation; see Toma et al., 2014) further supports their immature state. On the
441 other hand, the vast majority (around 90%) did not express NeuN, a soluble nuclear protein whose
442 immunoreactivity becomes obvious as neurons are initiating cellular differentiation (Mullen et al.,

443 1992), and only a few of them did express HuC/D. The small percentage of DCX+ cells which
444 express markers of differentiation/maturity (mainly those with complex morphology) are likely in a
445 state ready for further differentiation. Notably, in cortex and amygdala their morphology is
446 reminiscent of the principal cell type (Washburn and Moises, 1992). Hence, most of the DCX+ cells
447 appear to be in an intermediate state of immaturity (Fig. 8F). Theoretically, they may either be adult
448 “immature neurons” in standby mode, or adult neurons undergoing structural plasticity (i.e., a “de-
449 maturation and re-maturation” process akin to dedifferentiation and redifferentiation). Due to
450 obvious difficulties in performing functional experimental tests in sheep, these latter options remain
451 hypothetical. Yet, these results, including the fact that immature cell populations in subcortical
452 regions appear more stable over time with respect to their cortical counterpart, open new
453 possibilities for the existence of unusual/unknown forms of plasticity in multiple brain regions of
454 non-rodent mammals. The fact that “immature” cell populations in the sheep brain are not
455 regionally restricted as they are in rodents, confirms that non-neurogenic structural plasticity might
456 be higher in non-rodent species (Bonfanti and Nacher, 2012; Fig. 10). Since adult neurogenesis is
457 well preserved in rodents and highly reduced in species evolutionarily and structurally closer to
458 humans (Sanai et al., 2011; Paredes et al., 2015; Parolisi et al., 2015,2017; Lipp and Bonfanti,
459 2016), we suggest that non-neurogenic plasticity might have been preserved better in long-living,
460 large-brained mammals (though only studies through mammalian orders might identify putative
461 phylogenetic trends).

462 Markers of pallial/subpallial origin were used to get insights into the embryologic origin and, to a
463 lesser extent, the possible fate of the DCX+ cells described here. According to the tetrapartite model
464 of pallial subdivision in vertebrate brain, four main territories of progenitor domains can be
465 recognized in pallial germinative regions: medial, dorsal, lateral, ventral pallium (Holmgren, 1925;
466 Puelles et al., 2000). *Tbr1* is a neuron specific, post-mitotic transcription factor mostly found in
467 pallium-derived neurons committed to differentiate into excitatory glutamatergic neurons (Bedogni
468 et al., 2010; Puelles et al., 2000, McKenna et al., 2011). Most of the DCX+ cells described here in

469 cortical and subcortical regions expressed *Tbr1*, thus belonging to the glutamatergic principal
470 cortical cells of pallial origin (Hevner et al., 2001). Whereas in rodents the DCX+ immature
471 neurons are confined in the ventral pallial derivative (paleocortex), we show that immature neurons
472 in sheep extend into other ventral (amygdala), dorsal (neocortex) and lateral (claustrum) pallial
473 derivatives. In the external capsule and amygdala, part of the DCX+ cells expressed *Sp8*, a
474 transcription factor marking specific populations of olfactory bulb interneurons, strongly expressed
475 in the dorsal lateral ganglionic eminence (a subpallium domain; Waclaw et al., 2006) and in one-
476 fifth of adult cortical interneurons (Ma et al., 2011). We show that the vast majority of DCX+
477 cortical and claustrum neurons in sheep are of pallial origin, whereas capsular and amygdalar
478 DCX+ cells are of mixed origin (pallial and non-pallial sources). Hence, it is very likely that
479 capsular and peri-capsular immature neurons derive from different populations during
480 embryogenesis, though genetic lineage tracing would be required to confirm this. Since the anlage
481 of the external capsule is a migration route for *Tbr1*+ and *Sp8*+ cells directed to claustrum and
482 amygdala (Waclaw et al., 2006; Puelles et al., 2017), the DCX+ cells in subcortical regions might
483 represent a remnant of immature cells remaining in the white matter during postnatal brain growth.
484 As to the possible function of the DCX+ "immature" neurons, no substantial insight have been
485 obtained until now, even in rodents. In the rat paleocortex they are considered as a reservoir of
486 undifferentiated elements somehow kept in a "stand by" mode (Gomez-Climent et al., 2008). The
487 current hypothesis (utterly theoretical), is that they might lose immaturity at a certain point of life,
488 possibly integrating in the neural circuits by accomplishing their differentiation (Bonfanti and
489 Nacher, 2012). In brain regions hosting adult neurogenesis (olfactory bulb, hippocampus), young
490 neurons are endowed with special plastic properties enabling them to substantially affect neural
491 functions independently from their long-term integration (Stone et al., 2011; Ishikawa et al., 2014).
492 Thus, an intriguing possibility is that, in some mammals, other pallial regions may foster related
493 types of plasticity by extending the immature phase of specific neuronal subpopulations without the
494 need of increasing their number. In the cortex, immature *Tbr1*/DCX+ neurons gradually fade-off

495 during post-natal life (Abrous et al., 1997; Xiong et al., 2008; Cai et al., 2009; this study)
496 suggesting that they could be related to cortical maturation. The reason why the number of DCX+
497 cells in claustrum and amygdala remain strikingly constant during post-natal stages is puzzling.
498 These cells may represent a specific subpopulation that constitutively express DCX through life and
499 support a form of plasticity that is independent from the general trend of maturation-related
500 structural plasticity. All brain structures hosting the DCX+ immature neurons mediate high
501 cognitive functions, including learning, memory and emotional activities. The amygdala has an
502 essential role in the formation of emotion-related memories (LaBar and Phelps, 1998), whereas the
503 claustrum is considered important in consciousness (Crick and Koch, 2005). Finally, an important
504 point will be to understand the evolutionary relationships of the DCX+ cells. *Tbr1*+/*DCX*+ cells are
505 present in pallial derivatives of reptiles (including the dorsal ventricular ridge, a ventral pallial
506 derivative homologous of the amygdala; Puelles et al., 2017), leading to propose that they could
507 represent a conserved pallial cell type (Luzzati, 2015). Collectively, these observations support the
508 possibility that a population of slowly maturing DCX+ cells might be shared by multiple pallial
509 domains being conserved during evolution despite the profound functional/anatomical changes.
510 Independently from any specific function, “immature” neurons raise interest in the general context
511 of mammalian structural plasticity, representing an endogenous reserve of potentially plastic cells.

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757

758

759 **Figure legends**

760

761 **Figure 1.** A, Four levels of interest (L1-L4) in the whole sheep brain, obtained by re-drawing the
762 Atlas of Brain Biodiversity Bank (Michigan State University; *www.msu.edu*) and adapted to our
763 cryostat sections; the whole extension of the brain analysed is comprised between the arrowheads
764 (dotted line). B, By combining levels L2 and L3, an additional, "ideal" level containing the most
765 important neuroanatomical structures analyzed here was obtained (asterisk); this ideal level was
766 used to represent Results. This representation refers to adult animals, yet no significant
767 morphological/neuroanatomical differences were observed in younger animals. Nc, neocortex; Pc,
768 paleocortex; Ic, internal capsule; Ec, external capsule; Ex, capsula extrema; Cl, claustrum; Am,
769 amygdala; Pu, putamen; Cn, caudate nucleus; Lv, lateral ventricle; Cc, corpus callosum.

770

771 **Figure 2.** DCX+ cells in the adult sheep brain. A and C, representative level of the brain showing
772 different locations of the DCX+ cells. B, main types of DCX+ cells encountered in our analysis
773 (DCX+ "objects"), classified according to their morphology, spatial organization, and cell division
774 history (newly born Vs. non-newly generated). D, newly generated neuroblasts in the SVZ and
775 dentate gyrus (SGZ); in both neurogenic sites, DCX+ cells are intermingled with several nuclei
776 immunoreactive for Ki-67 antigen (rarely double-stained in the SVZ due to different expression
777 time-course of the markers); BrdU injected 60 days before sacrifice is detectable in DCX+
778 neuroblasts of the olfactory bulb (OB) and in hippocampal granule cells. E-G, representative
779 photographs of the DCX+ cells/cell populations at the different locations showed in C: E, clusters of
780 DCX+ cells in the external capsule (Ec); F, layer II cortical neurons; G, scattered DCX+ cells in the
781 amygdala (Amy) and claustrum (Cl); Ex, capsula extrema. Scale bars: 30 μm ; D (bottom right), 20
782 μm .

783

784 **Figure 3.** DCX+ cells in the cerebral cortex of adult sheep. A, Location of DCX+ neurons in the
785 cortical layer II. Top, DCX (brown) and cresyl violet staining; coronal section cut at the level of the
786 frontal lobe; layer IV (inner granular layer) is absent in the agranular isocortex of sheep (see Beul
787 and Hilgetag, 2015); WM, white matter. Bottom, confocal image of the first two cortical layers
788 (DCX, white; DAPI, blue). B, DCX+ neurons are present both in paleo- (piriform cortex) and neo-
789 cortex; arrows, type 1 neurons; arrowheads, type 2 neurons (see D). C,D, Main morphological types
790 of the DCX+ neurons (neocortex); type 1: small cell body and simpler apical dendritic arborization
791 (ad); type 2: large cell body and more elaborated apical dendritic arborization (type 2a), also
792 including basal dendrites (bd; type 2b); type 2 cells represent about 7% of total DCX+ cells. Scale
793 bars: 30 μm .

794
795 **Figure 4.** DCX+ cells in the external capsule of the adult sheep brain. A,C, Numerous clusters of
796 tightly-packed, DCX+ cells are present in most of the external capsule (EC). B, Serial
797 reconstruction showing their distribution and size. Ex, capsula extrema; Am, amygdala; Pu,
798 putamen; Cx, cerebral cortex. C, Examples of DCX+ cell clusters showing different types of
799 organization, spanning from large, tightly-packed cell masses to small groups of dispersed cells. E,
800 The cell clusters occupy empty spaces within white matter areas devoid of astrocytes. Scale bars: 30
801 μm .

802
803 **Figure 5.** DCX+ cells in the peri-capsular regions of the adult sheep brain; EC, external capsule;
804 Ex, capsula extrema; Cx, cerebral cortex. A, Groups of scattered, DCX+ cells are present within the
805 amygdala (Am; images in B) and claustrum (Cl, images in C). The morphology of the DCX+ spans
806 from small bipolar to large multipolar in the amygdala (B, bottom; in black, real drawing of some
807 cells; in brown, main cell types); it appears simpler in the claustrum, in which most cells are small
808 unipolar/bipolar and some show simple ramifications (C, bottom right). D, Quantification of the
809 relative amount of different cell types. Scale bars: 30 μm .

810

811 **Figure 6.** DCX+ cells in the sheep brain at different ages. A, Evident reduction of the amount of
812 DCX+ neurons in the cortical layer II with increasing age is clearly visible after qualitative analysis
813 (top). Quantitative evaluation of DCX+ cell linear density (number of DCX+ neurons in layer
814 II/cortical tract length; bottom) in three cortical regions (red areas) at two brain levels of the
815 newborn and adult sheep: Pc, piriform cortex; Ssg, suprasylvian gyrus; Ccx, cingulate cortex. B,
816 Striking reduction of DCX+ cell populations is clearly evident in the dentate gyrus (DG; note the
817 dilution of the DCX+ cell layer) and subventricular zone (SVZ; note the reduction in thickness of
818 the DCX+ germinal layer) neurogenic sites of neonatal and adult sheep. C, The occurrence,
819 morphology, distribution and amount (quantifications on the right; see also Table 3) of DCX+ cells
820 in the sheep capsular/pericapsular regions do not vary significantly at different ages (apart a slight
821 reduction observed in the external capsule, see text). Scale bars: 30 μ m.

822

823 **Figure 7.** DCX+ cells in cortical layer II, external capsule and peri-capsular regions of the adult
824 sheep are non-newly generated. A, Double staining with cell proliferation markers and DCX in the
825 brain parenchyma of the adult sheep: no double stained cells were found in any of the regions
826 investigated (same results with Ki-67 antigen; images not shown). B, BrdU and Ki-67 antigen
827 double staining with DCX in different brain regions of the neonatal brain (after BrdU treatment of
828 the ewes at the second month of pregnancy). Quantification of DCX+/BrdU+ cells in neonatal
829 lambs are represented in pie charts: populations of embryonically-generated cells are detectable
830 both in cortex and in capsular/pericapsular regions. Scale bars: 30 μ m.

831

832 **Figure 8.** Markers of neuronal maturity/immaturity in DCX+ cells of the adult (A,B,D) and young
833 (C,E) sheep brain. A, Double staining for DCX and NeuN (red arrows) in different brain regions;
834 EC, external capsule; Amy, amygdala; Cl, claustrum. B, Both in cortical (Cx) and in
835 capsular/pericapsular regions (Cps), the DCX+/NeuN+ cells represent a small subpopulation of all

836 DCX+ cells (red areas in pie charts); 1 and 2 in cortex pie chart refer to type 1/type 2 cells (see Fig.
 837 2). C, The marker of initial differentiation and maturity HuC/D is not detectable in the neurogenic
 838 sites (DG, dentate gyrus; SVZ, subventricular zone) and mostly absent in DCX+ immature neurons,
 839 apart from a weak expression in some type 2 cells of the cortical layer II (circle). D, Double staining
 840 with the marker of immaturity PSA-NCAM reveals all DCX+ cells largely decorated in the
 841 neurogenic zones (DG and SVZ), whereas only subpopulations of DCX+ cells are partially stained
 842 in the cortical and subcortical regions. E, Similarly to PSA-NCAM, the A3 subunit of the cyclic
 843 nucleotide-gated ion channel (CNGA3) is detectable in most DCX+ cells of the neurogenic sites
 844 and in subpopulations of DCX+ cells in other brain regions. F, Schematic summary of
 845 maturity/immaturity features in DCX+ cells of the sheep as revealed by different cellular markers
 846 (showed for the cortex but representative of all regions investigated). Scale bars: 30 μ m.

847

848 **Figure 9.** Origin of DCX+ cells by detection of pallial/subpallial markers in the neonatal sheep
 849 brain. A, Distribution of *Tbr1* and *Sp8* proteins in different DCX+ cell populations of the cerebral
 850 cortex, claustrum, external capsule and amygdala; DCX, white; *Tbr1*, purple; *Sp8*, red. Scale bars:
 851 30 μ m. B, Schematic summary of pallial (purple; SVZ counterpart of dorsal, ventral, lateral, medial
 852 pallium) and subpallial (yellow; lateral and medial ganglionic eminences) origin of the DCX+ cells
 853 in cortical (Cx) and capsular/pericapsular structures (Cps). Quantification results are reported in pie
 854 charts; most of the DCX+ cells in cortex and claustrum are only *Tbr1*+, whereas a mix of *Tbr1*+ and
 855 *Sp8*+ cell populations is detectable in the external capsule and amygdaloid nuclei.

856

857 **Figure 10.** Summary and comparative aspects. A, Two main populations of non-newly generated,
 858 "immature" DCX+ cells are present in the cerebral cortex and capsular/pericapsular regions of the
 859 sheep brain. B, Unlike newly generated neuroblasts of the main neurogenic sites and immature
 860 neurons of the cortical layer II, which are consistently reduced with age (see Fig. 5), the amount of
 861 DCX+ cells in the sheep capsular/pericapsular regions do not vary from neonatal to adult age. In

862 comparison with results reported for laboratory rodents, our findings in sheep strongly suggests that
863 parenchymal, non-neurogenic structural plasticity (brown areas) can be maintained/increased in
864 large brained, long living mammals, thus following an opposite trend with respect to adult
865 neurogenesis (green). C, Pallial and mixed (pallial and subpallial) origin of the DCX+ cells in
866 different brain regions.

867 **Tables**

868

869 **Table 1.** Animals used in the present study

870

Source	Sex	Breed	Age	BrdU treatment
INRA Nouzilly (France)	4 M, 3F	Ile-de-France	Neonate (7 days)	Survival: 97 days (injection: 90 days pre-partum)
	3 M		Prepuberal (4 months)	-
	9 F		Adult (2 years)	Survival: 120 days
				Survival: 60 days
				Survival: 30 days

871

872

873

874 **Table 2.** Primary antibodies used in this study

Antigen	Host	Dilution	RRID	Source
BrdU	Rat	1:300	AB_732011	AbDSerotec, Kidlington, UK
DCX	Rabbit	1:3000	AB_732011	Abcam, Cambridge, UK
	Goat	1:2000	AB_2088494	Santa Cruz Biotechnology, Santa Cruz, CA
	Guinea Pig	1:1000	AB_2230227	Abcam, Cambridge, UK
GFAP	Rabbit	1:800	AB_10013382	Agilent Technologies, Carpinteria, CA
	Mouse	1:200	AB_94844	Millipore, Bellerica, MA
Ki-67	Mouse	1:500	AB_393778	Agilent Technologies, Carpinteria, CA
NeuN	Mouse	1:1000	AB_177621	Millipore, Bellerica, MA
Sp8	Rabbit	1:10000	AB_877304	Millipore, Bellerica, MA
Tbr1	Rabbit	1:1000	AB_10806888	Chemicon, Temacula, CA
PSA-NCAM	Mouse	1:1400	AB_95211	Millipore, Bellerica, MA
HuC/D	Mouse	1:800	AB_221448	Invitrogen, Carlsbad, CA
CNGA3	Rabbit	1:250	AB_2039822	Alomone Labs, Israel

875

876

877

878

879 **Table 3.** Quantification of DCX+ clusters/cells in the capsular/pericapsular and cortical regions of
 880 neonatal and adult sheep brains

881

882 A

	Newborn			Adult		
	Area (mm ²)	Number of cells	Density (DCX+ objects/mm ²)	Area (mm ²)	Number of cells	Density (DCX+ objects/mm ²)
External capsule	1,72 ± 0,3	34 ± 4*	19,97 ± 1,4	4,28 ± 0,3	39 ± 7*	9,21 ± 1,6
Clastrum	7,52 ± 0,7	75 ± 22	9,78 ± 2,1	12,56 ± 1,2	77 ± 62	5,86 ± 4,3
Amygdala	11,44 ± 1,0	209 ± 75	18,44 ± 6,9	18,33 ± 3,5	252 ± 18	14,02 ± 2,1
	Length (mm)	Number of cells	Linear density (cells/mm)	Length (mm)	Number of cells	Linear density (cells/mm)
Neocortex	7,20 ± 0,4	329 ± 4	45,69 ± 2,6	9,47 ± 2,4	62 ± 15	6,54 ± 0,5
Paleocortex	4,46 ± 0,3	371 ± 11	83,41 ± 2,3	5,53 ± 0,8	164 ± 68	29,65 ± 7,2

883

884 B

	Newborn vs Adults T-tests p values		
	Area (mm ²)	Number of cells	Density
Two-way ANOVA	F=5,389; p=0.003	F=136,551; p<0,0001	F=84,258; p<0,0001
External capsule	<0.0001	0.300	0.001
Clastrum	0.003	0.961	0.231
Amygdala	0.030	0.422	0.347
	Number of cells	Length (mm)	Linear density (cells/mm)
Neocortex	<0.0001	<0.0001	<0.0001
Paleocortex	0.164	0.001	<0.0001

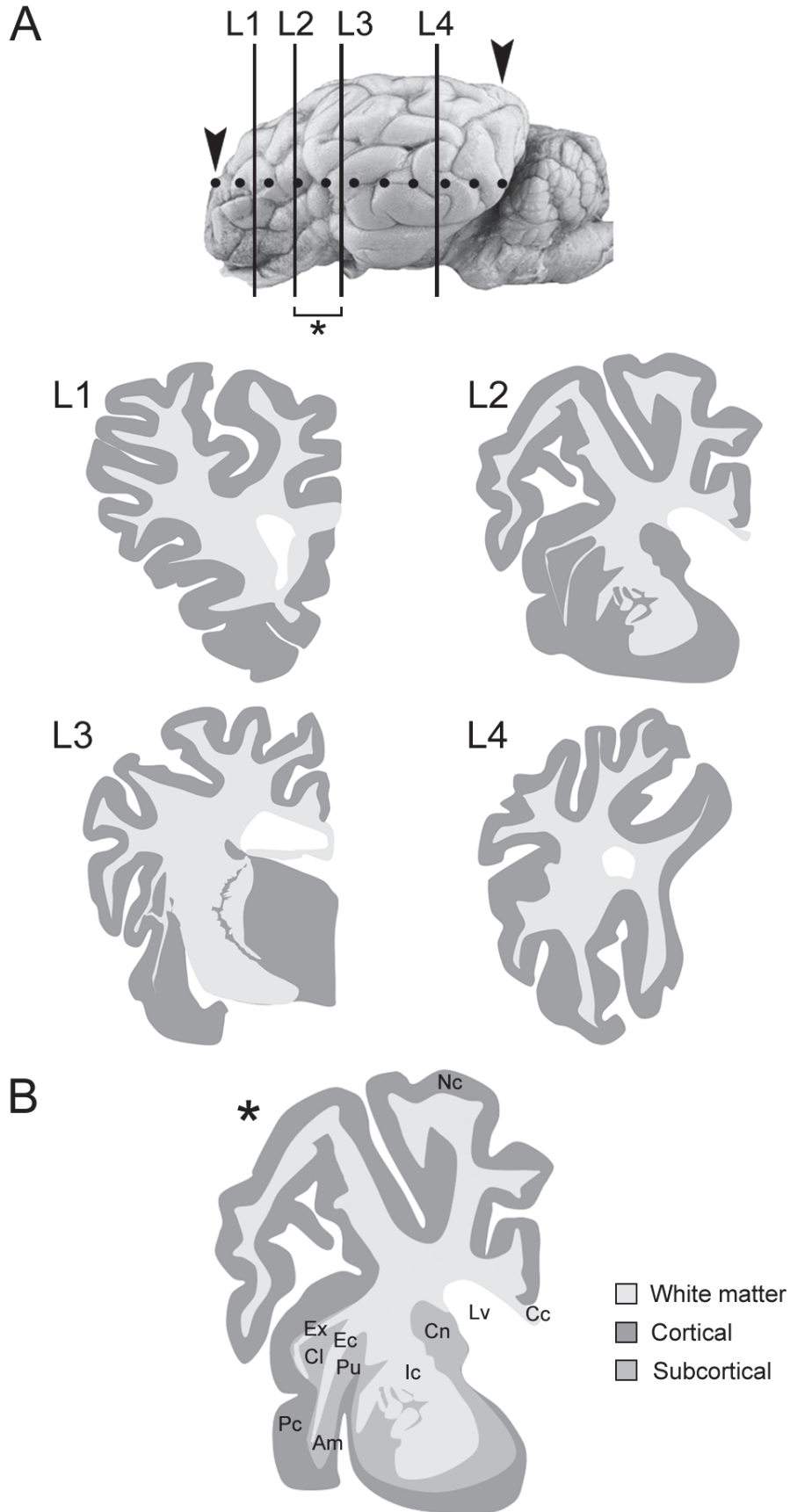
885

886 C

Number of cells/objects	Amigdala	Clastrum	EC*	Paleocortex	Neocortex
Amigdala		0.535	0.463	<0.0001	<0.0001
Clastrum	0.535		0.933	<0.0001	<0.0001
EC*	0.463	0.933		<0.0001	<0.0001
Paleocortex	<0.0001	<0.0001	<0.0001		<0.0001
Neocortex	<0.0001	<0.0001	<0.0001	<0.0001	
Density	Amigdala	Clastrum	EC*	Paleocortex	Neocortex
Amigdala		0.923	0.181	<0.0001	<0.0001
Clastrum	0.923		0.055	<0.0001	<0.0001
EC*	0.181	0.055		<0.0001	<0.0001
Paleocortex	<0.0001	<0.0001	<0.0001		<0.0001
Neocortex	<0.0001	<0.0001	<0.0001	<0.0001	

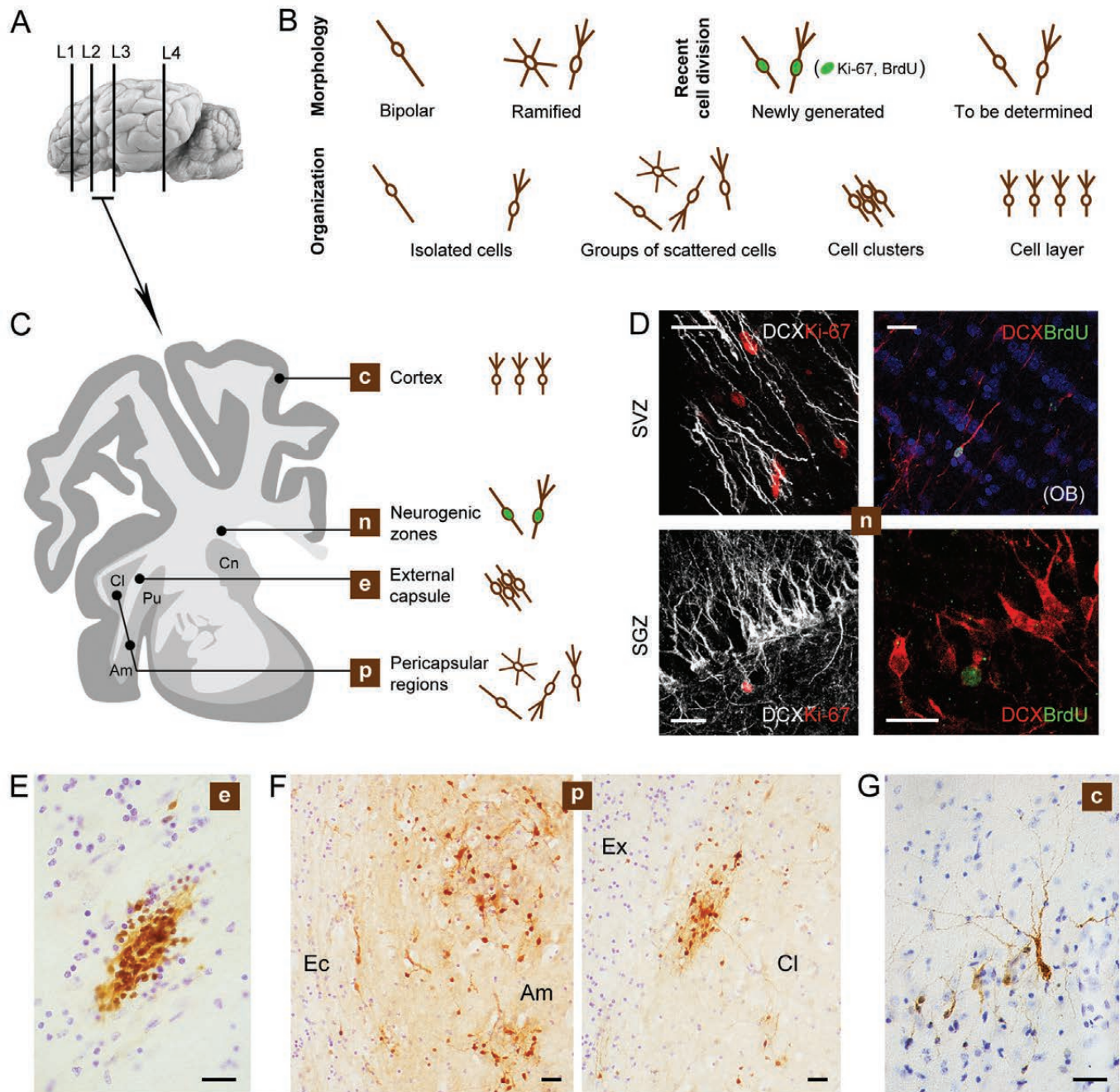
887 A, Number of DCX+ cells and density in cortical and subcortical regions. *DCX+ objects. B, Two-
 888 way ANOVA for all brain regions and T-tests for each region comparing newborn and adult values
 889 of perimeter/area, number and density of DCX+ cells. Numbers indicate p values, green and red fill

890 indicate p values lower or greater than 0.05 respectively. C, Pairwise two-way ANOVA analyses of
891 the density and number of DCX+ cells/objects in different brain regions between newborn and adult
892 animals. This analysis compares age related changes between pairs of brain regions, indicated in the
893 first row and column. Numbers indicate p-values for the interaction between age and brain region,
894 significant and non-significant interaction of these two factors are labelled in green and red
895 respectively. Yellow indicate a value that is close to the critical value of 0.05.



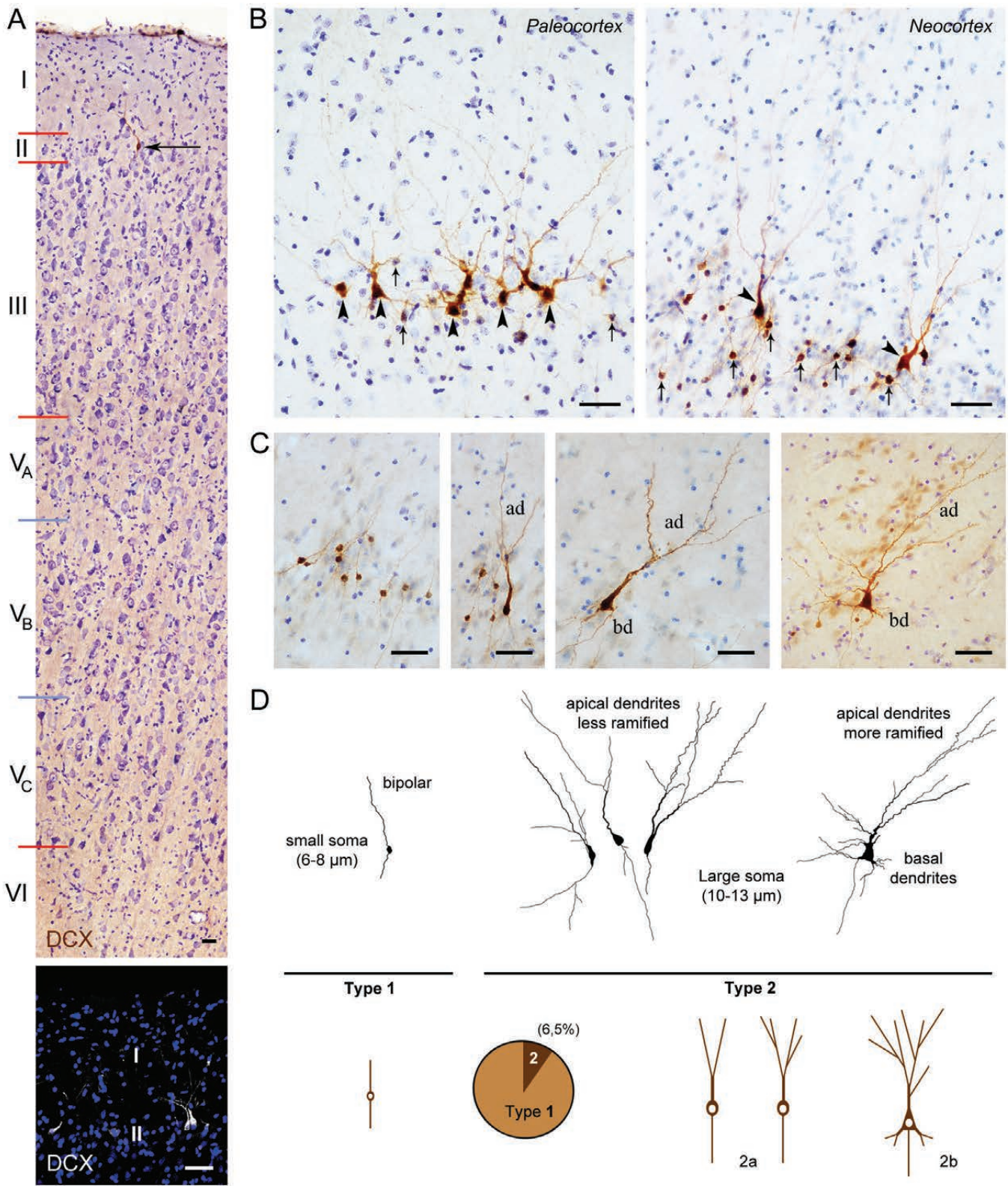
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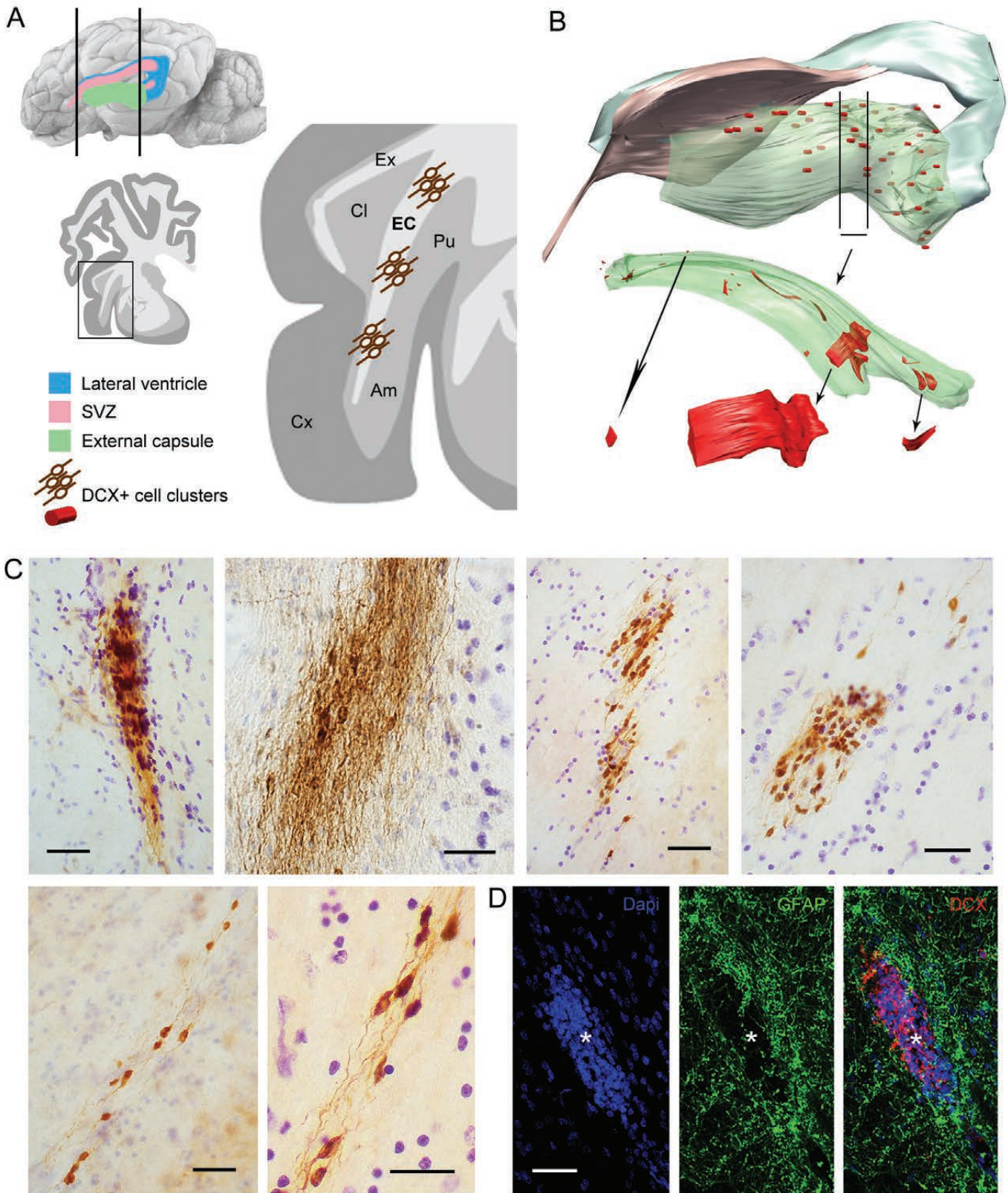
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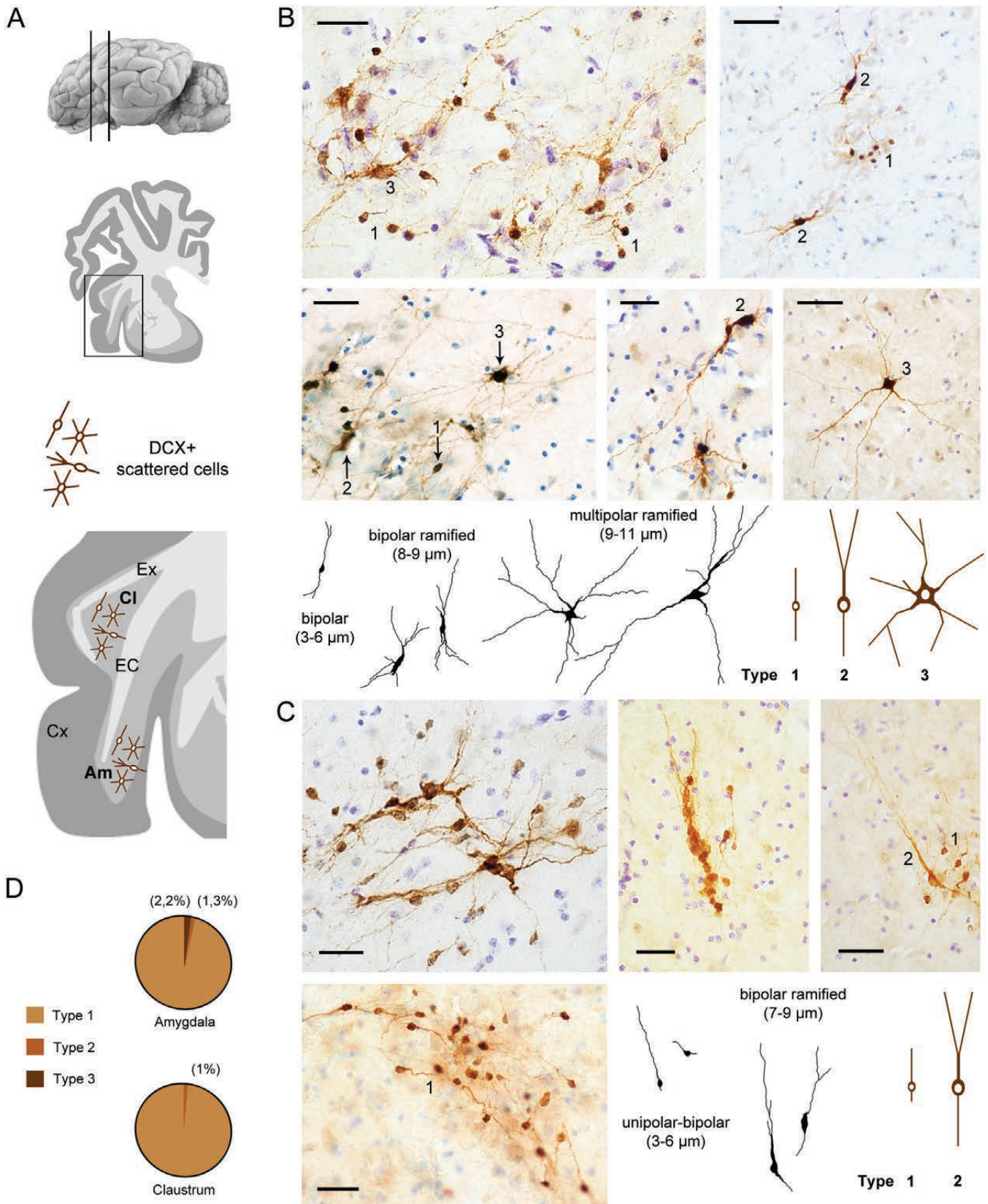
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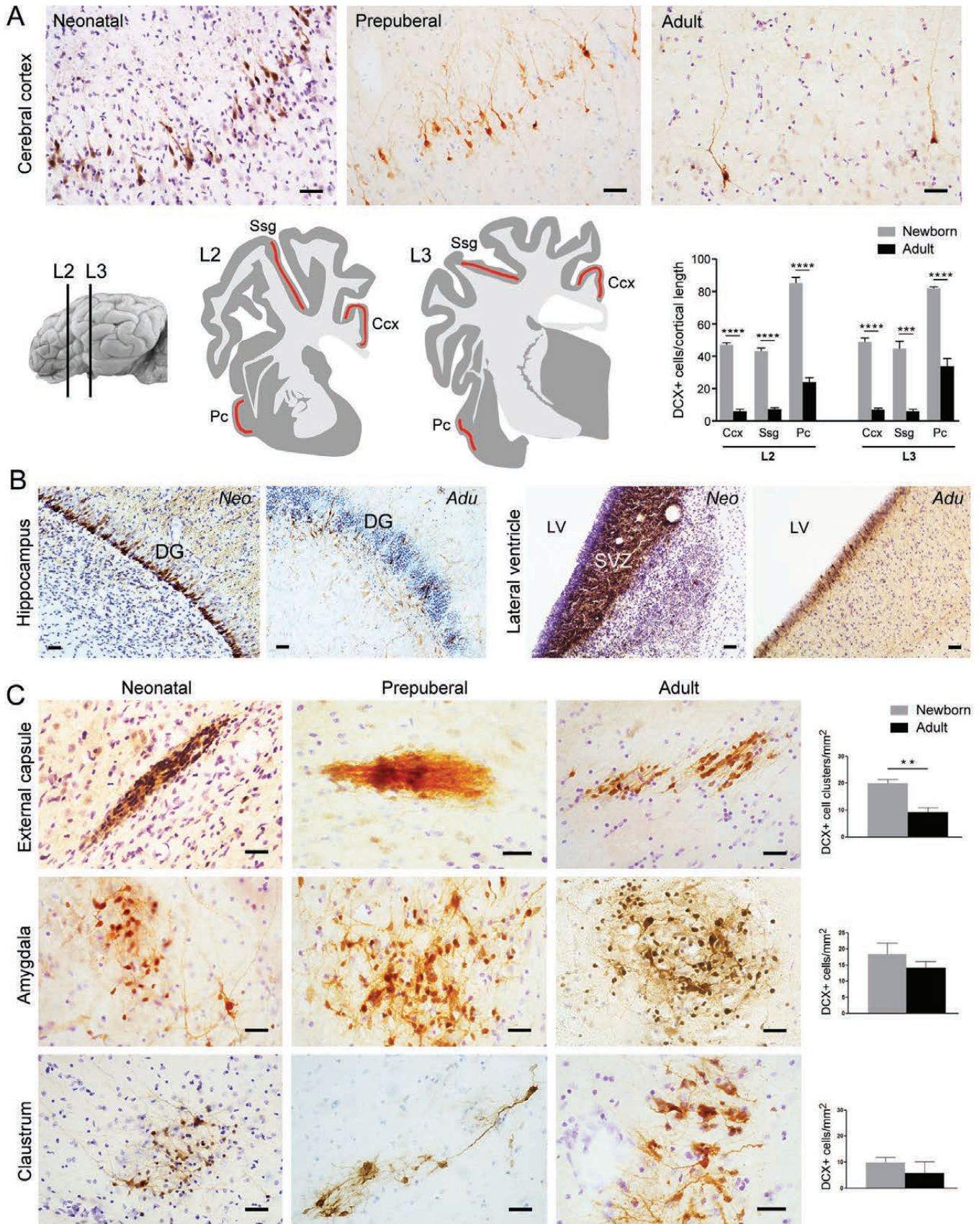
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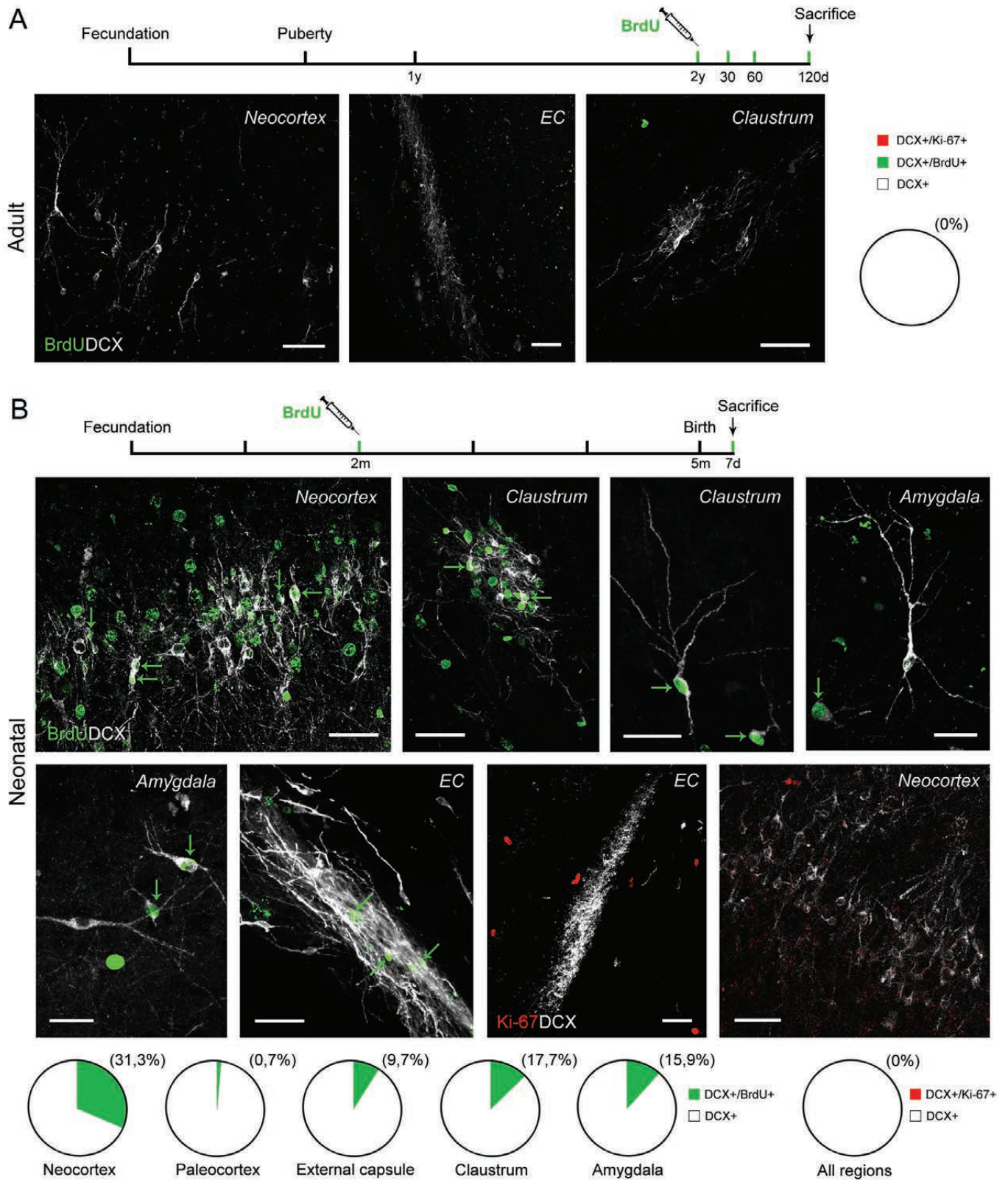
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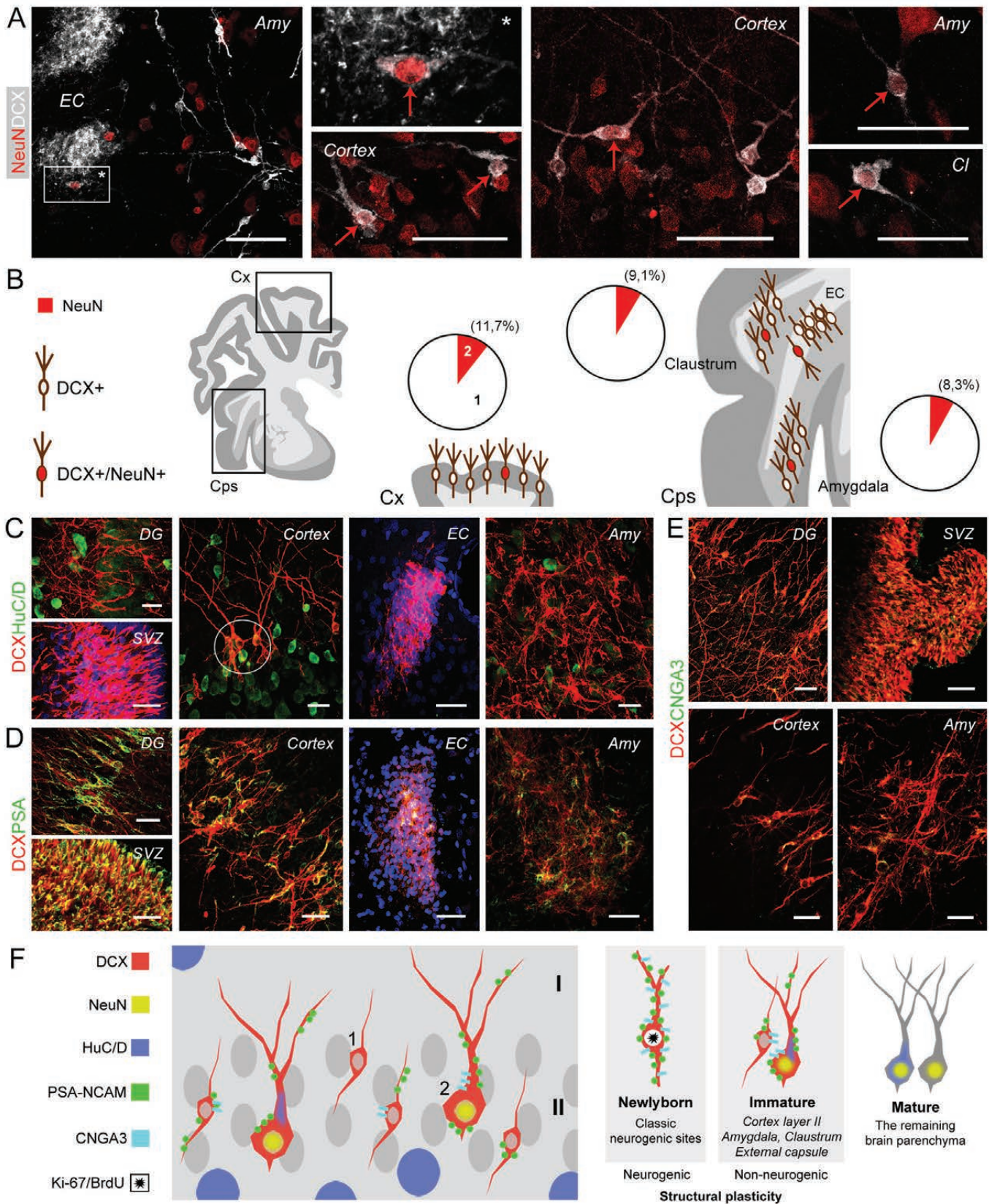
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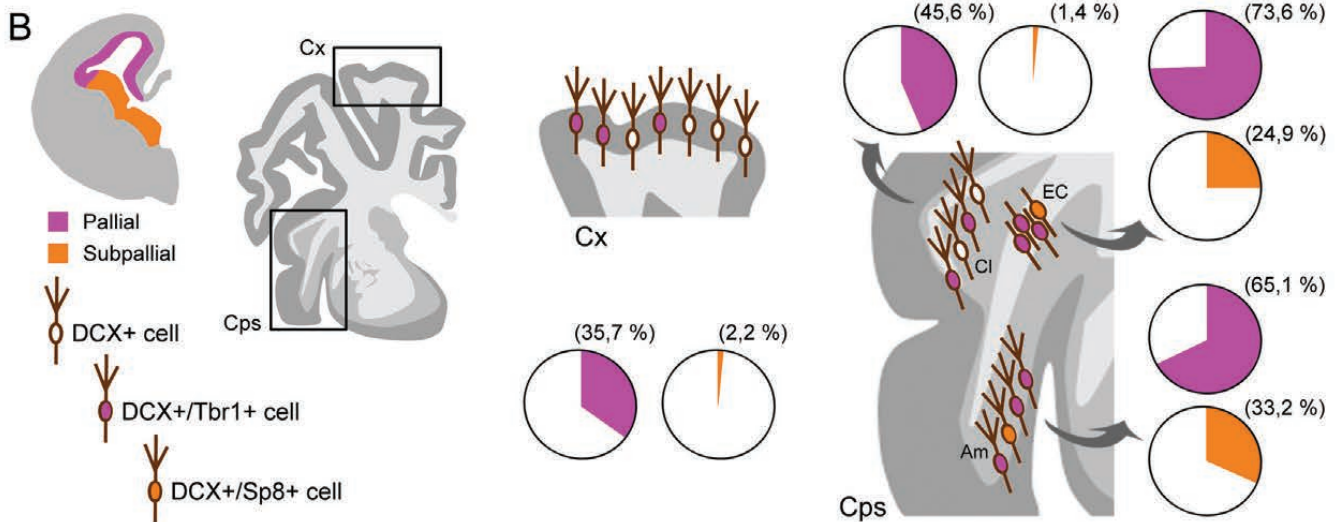
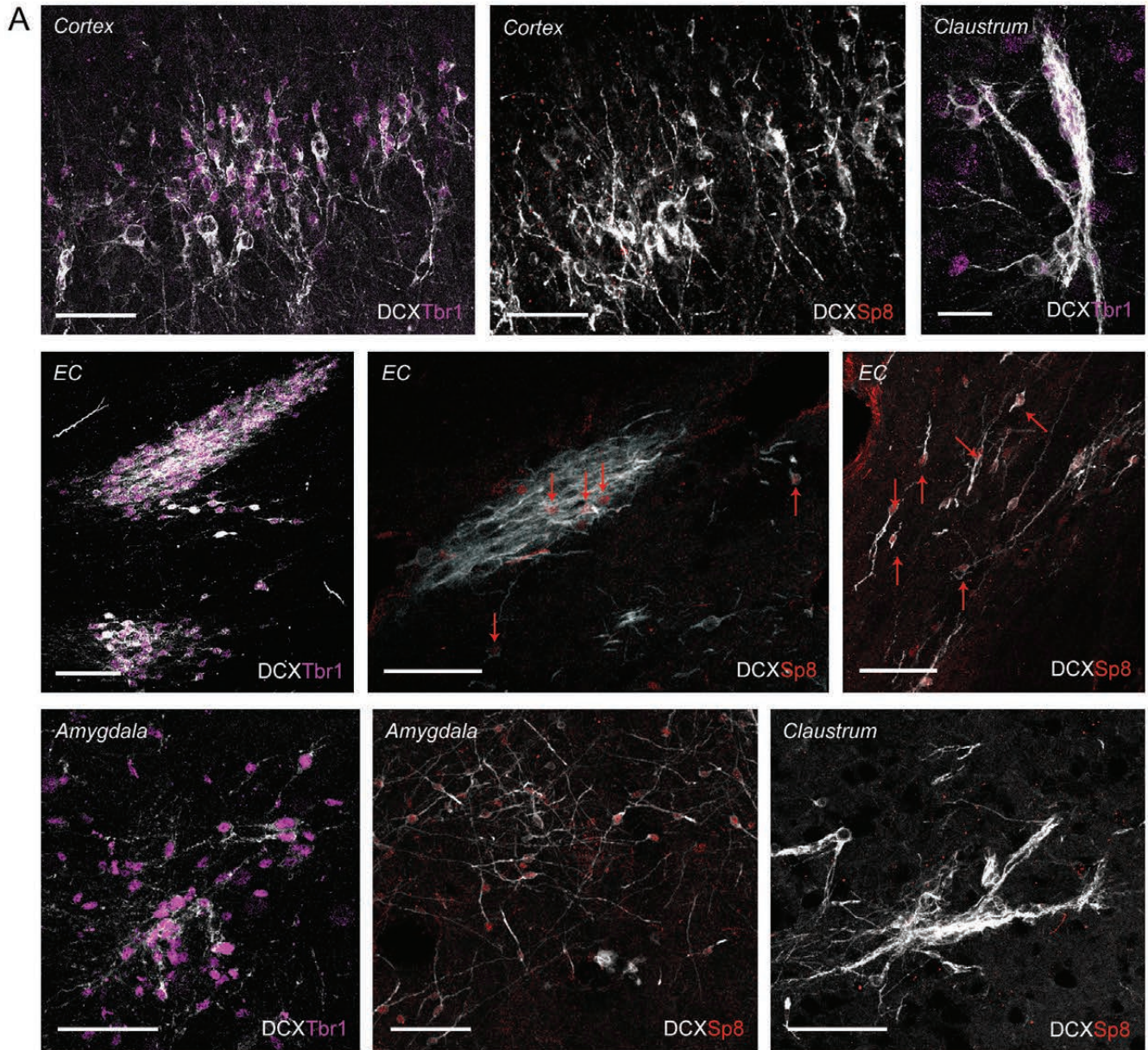
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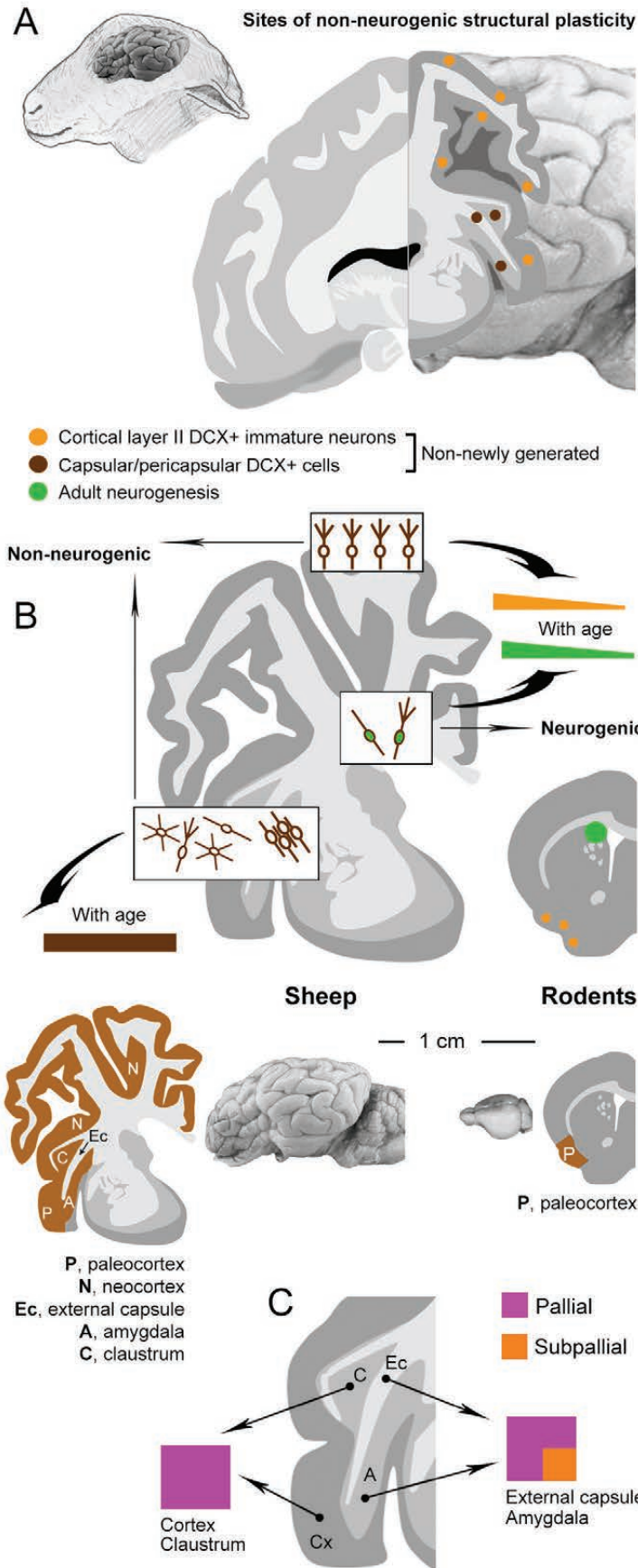
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