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1 **Non-newly generated, "immature" neurons**

2 **in the sheep brain are not restricted to cerebral cortex**

3

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

5

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Abstract

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

Significance statement

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

Introduction

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

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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.

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110 **Materials and Methods**

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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

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

3.2 Immunohistochemistry

Immunohistochemical reactions were performed on free-floating sections, when necessary, antigen retrieval was performed using citric acid at 90°C for 5-10 minutes. The section were incubated in 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-647conjugated donkey

152 anti mouse (1:800, Jackson ImmunoResearch, West Grove, PA), Alexa-647conjugated 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% H_2O_2 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

3.3 Quantitative analyses

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

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

Density of DCX+ objects in the external capsule at neonatal and adult ages: the number of DCX+ cell clusters and groups has been counted in the whole extension of the external capsule in 3 adult and 3 newborn animals, using a serial step of 12 cryostat sections (480 μm; 11 sections for the adult and 10 for the newborn). The density was calculated as the total number of objects/area (mm²). A 3-D reconstruction aimed at further characterize the DCX+ clusters in the adult has been made in the posterior part of the external capsule (24 serial cryostat sections, 40 μm thick, corresponding to 0,96 mm of white matter tissue).

Cell density in the amygdala and claustrum at neonatal and adult ages: the total number of DCX+ cells present within the two pericapsular regions have been counted in 3 adult and 3 newborn animals (three slices corresponding to the anterior, middle and posterior part of each anatomical structure have been considered). The density was calculated as the total number of cells/area (mm²).

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

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

12

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).

13

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

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

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%

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

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

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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.

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402

403   **Discussion**

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

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

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

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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 (clastrum) 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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Figure legends

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

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

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

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

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

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

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

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

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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 µm.

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 µm. 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

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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**

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

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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

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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

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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
Claustrum	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 (mm2)	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
Claustrum	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	Claustrum	EC*	Paleocortex	Neocortex
Amigdala		0.535	0.463	<0.0001	<0.0001
Claustrum	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	Claustrum	EC*	Paleocortex	Neocortex
Amigdala		0.923	0.181	<0.0001	<0.0001
Claustrum	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.

A

L1 L2 L3 L4

*

L1

L2

L3

L4

B

*

Nc

Ex
Cl   Ec
       Pu

Cn   Lv
              Cc

Ic

Pc
       Am

☐ White matter
⬛ Cortical
▨ Subcortical

A

L1 L2 L3 L4

B

Morphology

Bipolar   Ramified   Recent cell division (🟢 Ki-67, BrdU) Newly generated   To be determined

Organization

Isolated cells   Groups of scattered cells   Cell clusters   Cell layer

C

c Cortex

n Neurogenic zones

Cn

Cl   e External capsule

Pu

Am   p Pericapsular regions

D

SVZ   DCX Ki-67   DCX BrdU

(OB)

n

SGZ   DCX Ki-67   DCX BrdU

E

e

F

p

Ec   Am

Ex

Cl

G

c

A

I
II
III
V_A
V_B
V_C
VI

DCX

I
II

DCX

B   *Paleocortex*   *Neocortex*

C   ad   ad   ad

                                                                        bd   bd

D

apical dendrites
less ramified

apical dendrites
more ramified

bipolar

small soma
(6-8 μm)

Large soma
(10-13 μm)

basal
dendrites

Type 1   **Type 2**

(6,5%)

2

Type **1**

2a   2b

A

Lateral ventricle
SVZ
External capsule
DCX+ cell clusters

Ex
Cl
EC
Pu
Am
Cx

B

C

D

Dapi   GFAP   DCX

A

DCX+
scattered cells

Ex

Cl

EC

Cx

Am

B

bipolar
(3-6 μm)

bipolar ramified
(8-9 μm)

multipolar ramified
(9-11 μm)

Type 1 2 3

C

unipolar-bipolar
(3-6 μm)

bipolar ramified
(7-9 μm)

Type 1 2

D

(2,2%) (1,3%)

Type 1
Type 2
Type 3

Amygdala

(1%)

Claustrum

A

Neonatal | Prepuberal | Adult

Cerebral cortex

L2 L3

Ssg
L2
Ccx
Pc

Ssg
L3
Ccx
Pc

Newborn
Adult

DCX+ cells/cortical length

**** **** **** **** *** ****

Ccx Ssg Pc Ccx Ssg Pc
L2 L3

B

Hippocampus

Neo DG *Adu* DG

Lateral ventricle

Neo LV SVZ *Adu* LV

C

Neonatal | Prepuberal | Adult

External capsule

Newborn
Adult

DCX+ cell clusters/mm^2

**

Amygdala

DCX+ cells/mm^2

Claustrum

DCX+ cells/mm^2

A

Fecundation   Puberty   BrdU   Sacrifice

1y   2y   30   60   120d

Adult

Neocortex   *EC*   *Claustrum*

BrdU DCX

■ DCX+/Ki-67+
■ DCX+/BrdU+
□ DCX+

(0%)

B

Fecundation   BrdU   Birth   Sacrifice

2m   5m   7d

Neonatal

Neocortex   *Claustrum*   *Claustrum*   *Amygdala*

BrdU DCX

Amygdala   *EC*   *EC*   *Neocortex*

Ki-67 DCX

(31,3%)   (0,7%)   (9,7%)   (17,7%)   (15,9%)   (0%)

Neocortex   Paleocortex   External capsule   Claustrum   Amygdala   All regions

■ DCX+/BrdU+
□ DCX+

■ DCX+/Ki-67+
□ DCX+

A

Cortex (DCXTbr1)	Cortex (DCXSp8)	Claustrum (DCXTbr1)
EC (DCXTbr1)	EC (DCXSp8)	EC (DCXSp8)
Amygdala (DCXTbr1)	Amygdala (DCXSp8)	Claustrum (DCXSp8)

B

Pallial
Subpallial

DCX+ cell
DCX+/Tbr1+ cell
DCX+/Sp8+ cell

Cx

Cps

Cl
EC
Am

(45,6 %) (1,4 %) (73,6 %)
(24,9 %)
(35,7 %) (2,2 %) (65,1 %)
(33,2 %)

A Sites of non-neurogenic structural plasticity

- Cortical layer II DCX+ immature neurons ⎤ Non-newly generated
- Capsular/pericapsular DCX+ cells ⎦
- Adult neurogenesis

B

Non-neurogenic

With age

Neurogenic

With age

Sheep **Rodents**

— 1 cm —

P, paleocortex
N, neocortex
Ec, external capsule
A, amygdala
C, claustrum

P, paleocortex

C

Pallial
Subpallial

Cortex
Claustrum

External capsule
Amygdala