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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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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

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

Materials and Methods

Animals, BrdU injections, tissue preparation

Neonatal, prepuberal and adult animals (Ile de France) were raised at the INRA research center (Nouzilly; Indre et Loire, France). Experiments were conducted on 9 adult (females, 2 year old), 3 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

temperature, then incubated for 24-48 h at 4 °C in a solution of 0.01 M PBS, pH 7.4, containing 0,5-1% Triton X-100, 2% normal serum and the primary antibodies (see Table 2). Sections were then incubated with appropriate solutions of secondary antibody: Alexa-488 conjugated goat anti mouse (1:400, Molecular Probes, Eugene, OR), Alexa-488 conjugated goat anti rabbit (1:400, Molecular Probes, Eugene, OR), Alexa-488 conjugated donkey anti rat (1:400 Jackson ImmunoResearch, West Grove, PA), Alexa-555 conjugated goat anti mouse (1:800, Molecular Probes, Eugene, OR), Alexa-555 conjugated goat anti rabbit (1:800, Molecular Probes, Eugene, OR), Alexa-555 conjugated goat anti guinea pig (1:800, Molecular Probes, Eugene; OR), cyanine 3 (cy3) conjugated goat anti mouse (1:800, Jackson ImmunoResearch, West Grove, PA), cyanine 3 (cy3) conjugated goat anti rabbit (1:800, Jackson ImmunoResearch, West Grove, PA), cyanine 3 (cy3) conjugated donkey anti goat (1:800,Jackson ImmunoResearch, West Grove, PA), Alexa-647conjugated donkey anti mouse (1:800, Jackson ImmunoResearch, West Grove, PA), Alexa-647conjugated donkey anti rabbit, for 3h at RT. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, KPL, Gaithersburg, MD), coverslipped with MOWIOL 4-88 (Calbiochem, Lajolla, CA) and examined. For 3,3'-diaminobenzidine (DAB) immunohistochemistry sections were incubated in a solution of 0,3% H_2O_2 in 0,01 M PBS, ph 7.4 for 15 minutes to inhibit the endogenous peroxidase, before the incubation with blocking buffer. Following primary antibody incubation sections were incubated with goat anti rabbit IgG biotinylated secondary antibody (1:350, Vector Laboratories, Burlingame, CA) or horse anti goat IgG biotinylated secondary antibody (1: 250, Vector Laboratories, Burlingame, CA) for 2h at RT. Sections were washed and incubated in avidin–biotin–peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 2h at RT. The reaction was developed with DAB Peroxidase Substrate Kit, 3,3'-diaminobenzidine (Vector laboratories, Burlingame, CA) for 3-5 minutes. Sections were mounted on TESPA (3-aminopropyl-triethoxysilan) treated slides and then counterstained with 1% cresyl violet acetate solution (1-2 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).

Cell soma diameters of DCX+ cells: the average cell soma diameter (orthogonal to main axis) has been measured for 200 cells in the cortex, 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.

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

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

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

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

3.4 Image acquisition and processing

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

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

Results

Distribution of DCX+ cells in the adult sheep brain

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

DCX+ cells in the cerebral cortex

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

DCX+ cells in the external capsule and surrounding regions

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

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

External capsule. Most DCX+ cells in capsular white matter appeared as bipolar, elongated elements, often endowed with very long cell processes, assembled to form tightly-packed cell clusters (Fig. 4C). These clusters showed remarkable diameter variability (between 50 and 380 µm). When observed in single coronal sections, some of them were reminiscent of the thick chains of neuroblasts described in the SVZ of rodents (Lois et al., 1996) and in the surrounding regions of rabbits (Luzzati et al., 2003; Ponti et al., 2006). Hence, a 3D reconstruction was performed through an anterior-posterior portion of the external capsule (Fig. 4B; see Methods) in order to assess their relationship with the SVZ neurogenic site. The analysis revealed that all clusters appear as discrete bulks of cells not forming long streams and never contacting the SVZ neurogenic site. In order to investigate the relationship with surrounding cells (e.g., the occurrence of specific astrocytic arrangements), immunocytochemistry for glial fibrillary astrocytic protein (GFAP) was performed. GFAP staining did not revealed any special density or niche-like organization adjacent to the DCX+ cells (Fig. 4D); yet, when the DCX+ cells were organized to form tightly-packed clusters they occupied glial-empty spaces, the astrocytes being segregated outside them (Fig. 4D). Quantification of DCX+ cell clusters (see below and Table 3) revealed that virtually all of them were contained within the external capsule, mostly residing in its posterior part (Fig. 4B). This analysis further excluded any direct contact between the clusters and the SVZ neurogenic site. Groups of less tightly-packed cells and scattered/isolated cells were also observed (Fig. 4C).

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

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

DCX+ cell populations at different ages

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

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

Cell proliferation analysis

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

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

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

Cell maturity/immaturity and pallial/subpallial origin markers

In order to get some insight concerning the degree of maturation of the DCX+ cell populations described here, we used markers commonly employed to assess their neuronal maturational stage. We analysed NeuN, an RNA-binding protein expressed by most postmitotic neurons which start differentiation (Mullen et al., 1992; Fig. 8A). Only a small percentage of the DCX+ cells (around 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

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

Discussion

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

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.,

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

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

cortical and subcortical regions expressed *Tbr1*, thus belonging to the glutamatergic principal cortical cells of pallial origin (Hevner et al., 2001). Whereas in rodents the DCX+ immature neurons are confined in the ventral pallial derivative (paleocortex), we show that immature neurons in sheep extend into other ventral (amygdala), dorsal (neocortex) and lateral (clastrum) pallial derivatives. In the external capsule and amygdala, part of the DCX+ cells expressed *Sp8*, a transcription factor marking specific populations of olfactory bulb interneurons, strongly expressed in the dorsal lateral ganglionic eminence (a subpallium domain; Waclaw et al., 2006) and in one-fifth of adult cortical interneurons (Ma et al., 2011). We show that the vast majority of DCX+ cortical and claustrum neurons in sheep are of pallial origin, whereas capsular and amygdalar DCX+ cells are of mixed origin (pallial and non-pallial sources). Hence, it is very likely that capsular and peri-capsular immature neurons derive from different populations during embryogenesis, though genetic lineage tracing would be required to confirm this. Since the anlage of the external capsule is a migration route for *Tbr1*+ and *Sp8*+ cells directed to claustrum and amygdala (Waclaw et al., 2006; Puelles et al., 2017), the DCX+ cells in subcortical regions might represent a remnant of immature cells remaining in the white matter during postnatal brain growth.

As to the possible function of the DCX+ "immature" neurons, no substantial insight have been obtained until now, even in rodents. In the rat paleocortex they are considered as a reservoir of undifferentiated elements somehow kept in a "stand by" mode (Gomez-Climent et al., 2008). The current hypothesis (utterly theoretical), is that they might lose immaturity at a certain point of life, possibly integrating in the neural circuits by accomplishing their differentiation (Bonfanti and Nacher, 2012). In brain regions hosting adult neurogenesis (olfactory bulb, hippocampus), young neurons are endowed with special plastic properties enabling them to substantially affect neural functions independently from their long-term integration (Stone et al., 2011; Ishikawa et al., 2014). Thus, an intriguing possibility is that, in some mammals, other pallial regions may foster related types of plasticity by extending the immature phase of specific neuronal subpopulations without the need of increasing their number. In the cortex, immature *Tbr1*/DCX+ neurons gradually fade-off

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

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

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

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

Figure 10. Summary and comparative aspects. A, Two main populations of non-newly generated, "immature" DCX+ cells are present in the cerebral cortex and capsular/pericapsular regions of the sheep brain. B, Unlike newly generated neuroblasts of the main neurogenic sites and immature neurons of the cortical layer II, which are consistently reduced with age (see Fig. 5), the amount of 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

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

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

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

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	

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

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