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Clusters of DCX+ cells "trapped" in the subcortical white matter of early postnatal Cetartiodactyla (*Tursiops truncatus, Stenella coeruloalba* and *Ovis aries*)

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Abstract

The cytoskeletal protein doublecortin (DCX) is a marker for neuronal cells retaining high potential for structural plasticity, originating from both embryonic and adult neurogenic processes. Some of these cells have been described in the subcortical white matter of neonatal and postnatal mammals. In mice and humans it has been shown they are young neurons migrating through the white matter after birth, reaching the cortex in a sort of protracted neurogenesis. Here we show that DCX+ cells in the white matter of neonatal and young Cetartiodactyla (dolphin and sheep) form large clusters which are not newly generated (in sheep, and likely neither in dolphins) and do not reach the cortical layers, rather appearing "trapped" in the white matter tissue. No direct contact or continuity can be observed between the subventricular zone region and the DCX+ clusters, thus indicating their independence from any neurogenic source (in dolphins further confirmed by the recent demonstration that periventricular neurogenesis is inactive since birth). Cetartiodactyla include two orders of large-brained, relatively long-living mammals (cetaceans and artiodactyls) which were recognized as two separate monophyletic clades until recently, yet, despite the evident morphological distinctions, they are monophyletic in origin. The brain of Cetartiodactyla is characterized by an advanced stage of development at birth, a feature that might explain the occurrence of "static" cell clusters confined within their white matter. These results further confirm the existence of high heterogeneity in the occurrence, distribution and types of structural plasticity among mammals, supporting the emerging view that multiple populations of DCX+, non-newly generated cells can be abundant in large-brained, long-living species.

Introduction

The cytoskeletal protein doublecortin (DCX) is an excellent marker for cells that retain high potential for structural plasticity in the nervous system (Gleeson et al., 1999; Nacher et al., 2001). DCX is heavily expressed in newly generated neuroblasts and during the early phases of their migration/differentiation, being universally known and commonly used as an excellent marker for adult neurogenesis (Brown et al., 2003). Nevertheless, it is now clear that several types of neurons persistently express DCX through adulthood at different locations though they are generated prenatally (Gómez-Climent et al., 2008; Luzzati et al., 2009; Bonfanti and Nacher, 2012; Piumatti et al.,2018). Very little is known about these cells. They are thought to remain in an "immature" state for long periods, by persistently expressing markers of immaturity and possibly performing unknown types of structural plasticity (Bonfanti et al., 1992; Gómez-Climent et al., 2008; Bonfanti and Nacher, 2012; König et al., 2016; Piumatti et al., 2018). In the adult cerebral cortex, it has been proposed they could represent a "reserve" of young neurons progressively depleted through time to be recruited in cortical circuits after completing maturation (Nacher et al., 2001; Bonfanti and Nacher, 2012; König et al., 2016). We recently showed that these DCX+, non-newly generated cells could be widely present in relatively large-brained, long-living mammals (e.g., sheep), also extending in subcortical regions (Piumatti et al., 2018). On the whole, multiple evidences confirm that DCX is not strictly linked to adult neurogenesis, revealing potential differences in parenchymal structural plasticity among mammals, especially considering large, long-living species with respect to the small-brained, short-living rodents (Bonfanti and Nacher, 2012; Bonfanti, 2016). Some of the DCX+ cell populations of unknown function have been described in the postnatal brain white matter of different mammalian species (Luzzati et al., 2003; Ponti et al., 2006; Piumatti et al., 2018), including primates and humans (Fung et al., 2011; Paredes et al., 2016). In mouse and humans, the current explanation for these cells is that they are young neurons generated postnatally and migrating to the cortex (Inta et al., 2008; Le Magueresse et al., 2011, 2012; Riccio et al., 2012; Paredes et al., 2016).

Here we investigated the occurrence and features of DCX+ cells in two groups of Cetartiodactyla (sheep and dolphin), including two mammalian orders which were recognized as two separate monophyletic clades until recently (Artiodactyla, even-toed ungulates, and Cetacea, highly specialized for an aquatic lifestyle; Fig. 1A). Though they are superficially quite different (Gatesy et al., 1999; Murphy et al., 2004), molecular evidence and multiple data sets suggests that Cetacea evolved from artiodactyl ancestors (Graur and Higgins, 1994; Gatesy et al., 1999). Hence, despite the morphological distinctions between extant cetaceans and artiodactyls, the order Cetartiodactyla is evolutionarily monophyletic in origin, having diverged from the shared common ancestor with the nearest groups (Perissodactyla and Carnivora) around 90 million years ago (Murphy

et al., 2004). Cetartiodactyla are relatively large-brained, long-living mammals. Most species live for at least a decade in the wild, and captivity usually prolongs life (up to 30 years in sheep and 50-60 years in dolphins). The species considered here, including both terrestrial and aquatic Cetartiodactyla (*Tursiops truncatus, Stenella coeruloalba, Ovis aries*; Fig. 1B), share some brain features: highly folded cerebral cortex with relatively low neuronal density (Kazu et al., 2014; Poth et al., 2005; Kern et al., 2011) and an advanced stage of development at birth (Ridgway, 1990; McIntosh et al., 1979; Parolisi et al., 2015). We show that DCX+ cells form large clusters in the subcortical white matter of neonatal and young Cetartiodactyla. Unlike previous reports of postnatal addition of DCX+ neurons in the cortex of mice and humans, the cells in these clusters are not newly generated and do not reach the cortical layers, appearing "trapped" in the white matter tissue.

Materials and methods

Tissue samples and processing

Dolphin brains (*T. truncatus*, neonatal and adult; *S. coeruloalba*, young; see Table 1) were obtained from the Mediterranean Marine Mammal Tissue Bank (MMMTB) of the University of Padova at Legnaro, Italy. The MMMTB is a CITES recognized (IT020) research center and tissue bank, sponsored by the Italian Ministry of the Environment and the University of Padova, with the aim of harvesting tissues from wild and captive cetaceans and distributing them to qualified research centers worldwide. Post-mortem delay before actual sampling varied between 18 and 40 hours, always remaining in the range 2-3 according to the carcass classification (Geraci and Lounsbury, 2005; intervals for each specimen are indicated in Table 1). Brain coronal slices (see Parolisi et al., 2015) approximately 1-1.5 cm thick were collected during post-mortem procedures performed in the necropsy room of the Department of Comparative Biomedicine and Food Science of the University of Padova at Legnaro, and fixed by immersion in 4% formalin in phosphate buffer saline (PBS). Sheep brains were obtained from 9 adult pregnant ewes (2 year old), 3 young ewes (4 month old), and 6 neonatal lambs (1 week old; see Table 1) raised at the INRA research center (Nouzilly; Indre et Loire, France). Young ewes were housed together in a large pen. Adult ewes were housed in an individual pen during pregnancy (2x1 m); adults 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. These mothers were injected 3 months before parturition (i.e. at 2-month gestational days)

because the peak of DCX expression in sheep treated with BrdU is 3 months post-injection (Brus et al., 2013a,b). Three different survival times were analyzed in these adult animals: 1, 2 and 4 months. The survival times were chosen because in sheep the range of maturation time for neuroblasts is far longer than in mice, between 1 and 4 months (Brus et al., 2013a,b). 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 2 L of 1% sodium nitrite in phosphate buffer saline to ensure a good vasodilation and to remove blood cells, followed by 4 L of ice-cold 4% paraformaldehyde solution in 0.1 M phosphate buffer at pH 7.4. The brains were then dissected out, cut into blocks and post-fixed in the same fixative for 48 h. To assure a better cryoprotection, dolphin slices and sheep hemispheres have been cut to obtain smaller block: cubes (about 1.5x2.5 cm) have been achieved from thick, formalin-fixed dolphin slices (Parolisi et al., 2015); sheep hemispheres have been cut into 4 coronal slices (about 1.5 cm thick). Then, the tissues were washed in 0.1 M phosphate buffer (PB), pH 7.4, for 24 - 48 hours, then cryoprotected in graded concentrations of sucrose solutions up to 30% in 0.1 M PB, embedded in OCT (optimum cutting temperature, Bio-Optica), subsequently frozen by immersion in isopentane, and stored at -80°C. Cryostat cut dolphin and sheep sections (40 µm thick) were collected on glass slides treated with 3-Aminopropyltriethoxysilane (Sigma-Aldrich, 741442) and as free-floating sections respectively, then processed for immunohistochemistry and immunofluorescence. Cryostat sections from mouse brain were used for Gallyas stain and antibody controls (neonatal and 3 months old C57BL/6 mice raised at NICO; courtesy of Enrica Boda).

Assessment of comparable neuroanatomy

Dolphin and sheep brains differ in terms of brain size, weight, gyrencephaly index and overall neuroanatomical organization. For this reason, four comparable levels of interest were identified which include the main neuroanatomical regions in both species (Fig. 1C). By using the front face of the thick brain slices (see Parolisi et al., 2015), we obtained a graphic representation of the four brain levels (L1-L4) for each species: once photographed and imported in Power Point, the outlines of each coronal section, including the external surface, white/gray matter limits, and basal ganglia limits were drawn. To make the four levels comparable among species, the slices containing specific neuroanatomical structures of reference were chosen: L1, prefrontal cortex and opening of the lateral ventricle; L2, starting of internal and external capsulae; L3, starting of the claustrum and amygdala;

L4, occipital cortex and closing of the lateral ventricle. Each brain level can be considered a representation of a specific region (an ideal thick slice) in which we found the neuroanatomical structures of reference, in order to create a sort of atlas for comparison of the different brains in Cetartiodactyla.

Immunocytochemistry and Gallyas staining

Single and double staining for cell proliferation analysis (Ki-67 antigen, Kee et al., 2002, and BrdU, Piumatti et al., 2018), neuronal and glial antigens linked to immature and newly generated neurons (DCX, PSA-NCAM, NeuN, GFAP; Mullen et al., 1992; Bonfanti, 2006; Bonfanti and Nacher, 2012; Piumatti et al., 2018), embryonic origin (the T-box transcription factor Tbr1, marker of pallial origin; Puelles et al., 2000), GABAergic interneurons (GAD67, glutamic acid decarboxylase; Le Magueresse et al., 2011) or mature excitatory neurons (CAMKII; Varea et al., 2011), and molecular markers of synapses (Synaptophysin, Syp, Wiedenmann and Franke, 1985; VGAT, Chaudhry et al., 1998) were performed (see Table 2). For immunocytochemical analysis, two different protocols of indirect staining were employed: peroxidase (for cell cluster counts and diameter measurement) or immunofluorescence techniques (for phenotypic characterization and BrdU+/DCX+, Ki-67+ antigen cell counts). Both protocols were used in all animal species with no substantial variations, apart from normal serum, BSA and Triton X-100 dilutions. Different primary antibody dilutions indicated in Table 2 are referred to peroxidase or immunofluorescence staining. For 3,3'-diaminobenzidine (DAB) immunohistochemistry sections were incubated in a solution of 0.3 - 1% H_2O_2 in 0.01 M PBS, pH 7.4 for 15 - 20 minutes to inhibit the endogenous peroxidase, and then pre-incubated in blocking buffer (2 - 3% normal serum (NS), 1 - 2% bovine serum albumin (BSA), 1% Triton X-100 in 0.01 M PBS, pH 7.4) for 1 h at room temperature to reduce non-specific staining. Then sections were incubated for 48 h at 4°C in a solution with primary antibody (2 % NS, 1% BSA, 0.5% Triton X-100, primary antibody in a solution of 0.01 M PBS, pH 7.4). After incubation with primary antibodies (Table 2), sections were incubated with biotinylated secondary antibody (goat anti rabbit IgG - 1:350, Vector Laboratories, Burlingame, CA and horse anti goat IgG - 1:250, Vector Laboratories, Burlingame, CA). Immunohistochemical reactions were performed by the avidin–biotin–peroxidase method (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA, USA) and revealed using 3,3′-diaminobenzidine (3% in Tris-HCl) as chromogen. Sections were counterstained with Cresyl violet staining, according to standard procedures currently employed in our lab (see Ponti et al. 2006), mounted with Neo-Mont 109016 (Merck, Darmstadt, Germany).

In immunofluorescence staining the sections were rinsed in PBS and antigen retrieval was performed using citric acid at 90°C for 5-10 minutes to detect nuclear antigens (Taylor et al., 1994). Then slices were pre-incubated in blocking buffer (2 - 3% NS, 1 - 2% BSA, 1 - 2% Triton X-100 in 0.01 M PBS, pH 7.4), for 1h at room temperature. Then the sections were incubated for 48 h at 4°C in a solution of 0.01 M PBS, pH 7.4, containing 2% NS, 1% BSA, 1 - 0.5% Triton X-100 and the primary antibody. Following primary antisera incubation, sections were incubated with appropriate solutions of secondary cyanine 3 (Cy3)-conjugated, Alexa 488-conjugated, Alexa-555-conjugated and Alexa 647-conjugated antibodies (1:800; Jackson ImmunoResearch, West Grove, PA), for 4 hours RT. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, KPL, Gaithersburg, Maryland USA), mounted with MOWIOL 4-88 (Calbiochem, Lajolla, CA).

Cryostat sections of newborn dolphin, sheep and mouse were processed for standard Gallyas stain of myelin using silver nitrate: this stain, using 0.1% ammoniacal silver nitrate solution (pH 7.5), marks myelin and suppresses the production of silver by other elements through a mix of pyridine and acetic anhydride (Gallyas, 1979).

Image acquisition, processing and data analysis

The immunolabeled specimens were examined using a Nikon Eclipse 90i (Nikon, Melville, NY) microscope connected to a color CCD Camera, a Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany) and a Nikon Eclipse 90i (Nikon, Melville, NY) confocal microscopes. All 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.

Quantitative analyses, including cell counting and measurement, were performed using Neurolucida software (MicroBrightfield, Colchester, VT). The density of DCX+ cell clusters in subcortical white matter was evaluated in 3 newborn dolphins, 3 newborn and 3 young ewes, using a serial step of 1400 μm for dolphin and 480 μm for sheep. For each immunostained section, the contour corresponding to the subcortical white matter area was drawn and all the DCX+ clusters herein were marked with specific symbols available in Marker's toolbar: in this way, the cluster density was calculated (clusters/mm^2). The diameter (thickness orthogonal to main axis) of the DCX+ clusters and the distance between them and the SVZ region were measured using the Neurolucida "Measure line" tool in the same animals in which the cluster density was evaluated. The closest clusters to the SVZ external limit in sheep or the SVZ-like region in dolphin were considered; for levels anterior and posterior to the SVZ extension the distance from the ventricular wall was measured (thus

underestimating the distance from the SVZ). The average areas occupied by the clusters (pink areas in Figs. 3-5) were obtained by superimposing (in different Photoshop levels) the sections used for the localization and quantification of the clusters, and connecting the more external clusters (showed in Fig. 3B). The number of Ki-67+ nuclei was counted in each brain level of newborn animals within 3 square boxes of 1 mm^2 (2 boxes in the area occupied by the DCX+ clusters, and one in white matter regions devoid of clusters). The percentage of DCX+/BrdU+ double-stained cells has been calculated by analyzing 10 clusters in the subcortical white matter of 3 newborn ewes (a total number of about 1000 DCX+ cells).

All the graphs were designed using Graph Pad Prism (San Diego California, USA). Statistical analyses were performed by Graph Pad Prism software and included one-way ANOVA (to test the difference in population based on one characteristic or factor: cluster density in neonatal animals of each species - dolphin and sheep) and two-way ANOVA (to test comparison between populations based on multiple characteristics: cluster density in neonatal and young sheep). $p < 0.05$ was considered as statistically significant. Data are expressed as averages ± standard deviation (SD).

Results

Large DCX+ cell clusters in dolphin and sheep white matter: morphology, topographical distribution and phenotype

After evaluating the entire white matter in the brain of newborn, young and adult cetartiodactyla, a high number of clusters composed of small, tightly packed and mostly DCX+ cells was detected in newborn and young animals (Figs. 2-5). In dolphin, the clusters were present in subcortical white matter (here intended in its peri-ventricular and dorsal parts), external and internal capsule, while in sheep they were found in subcortical white matter, corpus callosum and external capsule (the latter previously described in Piumatti et al., 2018). Dolphin clusters appeared mostly radially-oriented, surrounding the subventricular zone-like region (see also Parolisi et al., 2017), whereas their orientation was mainly tangential to the SVZ in sheep. Measurement of cluster's thickness (maximum transversal diameters; see Fig. 2) revealed that they are thinner in newborn dolphins (about 15-25 μm, up to 50 μm) than in newborn ewes (40-50 μm, up to 80 μm), involving at least 5-15 or 8-30 cells, respectively (Fig. 2 and Table 3). The clusters were even larger in young sheep (40-80 μm, up to 100 μm), which also displayed larger white matter tracts with respect to neonates (see Table 3). In

both species, some DCX+ cell clusters were surrounded by GFAP+ astrocytic cells and processes, thus filling a space devoid of glia (Fig. 2). In dolphins, some clusters were lined by long processes, reminiscent of radial glia (Fig. 2A).

Considering adult animals of both species, the DCX+ clusters were absent in most of the white matter. Some differences were observed: in dolphin, only sporadic clusters were detectable (in some animals), while in sheep they disappeared from most of the white matter, enduring only in the external capsule (described in Piumatti et al., 2018). In each Cetartiodactyla species evaluated, the DCX+ cell clusters showed different topographical distribution at different brain levels (Figs. 3, 4 and Table 4). In sheep, they were present mainly in L1, decreasing in L2 and L3, and being absent in L4 (Fig. 4B). In dolphin, the clusters showed an opposite distribution, with a few DCX+ clusters in L1 and increasing numbers at posterior levels (mainly in L3; Fig. 3C). On the whole, the clusters occupied mostly the anterior part of the brain in sheep whereas they largely extended to the middle part in dolphins (see also the synthesis showed in Fig. 10).

In some areas of the white matter of neonatal and young animals of both species analyzed, in which the cluster density was high (especially L1 and L2 for sheep and L2 and L3 for dolphin), they were mixed with isolated DCX+ cells, mainly with bipolar morphology (not shown). In all sections, the clusters were never saw to reach the cerebral cortex, always remaining within the white matter (see Figs. 3, 4 and 7).

Cell proliferation analysis

In both dolphin and sheep brains, at all ages examined, no proliferating cells (Ki-67+) were detectable within the DCX+ cell clusters and/or in association with isolated DCX+ cells in the white matter. In all animals, only rare Ki-67+ cells were occasionally detectable in the white matter surrounding the clusters and filling the spaces among them (Fig. 6). Their amount was very low in both species, with average densities (cells/mm^2) of 2,95 ± 2,17 in neonatal dolphin and 4,89 ± 1,25 in neonatal sheep (quantitative analysis at the different brain levels showed in Table 5). In lambs born from ewes injected with BrdU during pregnancy (3 months before birth), all DCX+ cell clusters showed high rates of co-localization (about 60%; Fig. 6). Taken into account that BrdU treatment had been administered in a short time window (see Methods and Fig. 6), the results confirm that most DCX+ cells in the clusters were generated during embryogenesis.

Spatial relationship between DCX+ clusters, SVZ region and cortex

Due to the shape and orientation of the DCX+ cell clusters, somehow reminiscent of migratory streams of neuroblasts (in dolphin also including radial glia-like remnants), the periventricular white matter was systematically analyzed at all anterior-posterior brain levels in search for continuity between the clusters themselves and the SVZ neurogenic site or the cortical layers (Fig. 7). After analyzing 50 sections for sheep and 100 sections for dolphin, spanning through all brain levels (see Table 6), direct connections with the SVZ area were never detectable. In all cases (newborn and young animals of both species), a continuous gap devoid of clusters and/or cells was consistently present, measuring about 2500 μm in dolphin and 1100 μm in sheep (Figs. 7 and 9; Table 6). Similarly, on the external (cortical) side, no DCX+ cells or clusters were detectable within the deep cortical layers (Fig. 7). Rare, isolated DCX+ cells were occasionally observed close to the white matter/cortex limit, always located on the white matter side (see Fig. 7B,C). As expected, the DCX+ immature neurons of the cortical layer II were observed to form their characteristic monolayer beneath the molecular layer (close to the cortical surface; Fig. 7C). Yet, these cells are known to be generated before birth (Gómez-Climent et al., 2008; Piumatti et al., 2018).

Further characterization of DCX+ cell clusters in sheep

Higher availability of neonatal and young animals and higher number of antibodies working in sheep allowed further investigation of phenotypic features of the cell clusters in this animal species (Fig. 8). In neonatal sheep, most DCX+ cells in the clusters were also expressing PSA-NCAM (Fig. 8 A1) whereas the co-expression was detectable only in a few cells in young animals (Fig. 8 A2). By contrast, substantially no DCX+ cells were immunopositive for NeuN (Fig. 8 A3; apart from occasional isolated cells at the periphery of the clusters; not shown) at any ages examined. Most DCX+ cells in the clusters were co-expressing Tbr1 (Fig. 8 A4), thus revealing their pallial origin. No immunoreactivity for GAD67, neither for CAMKII was detectable in association with any of the DCX+ cells and clusters (staining and positive controls in Fig. 8 B). Virtually no synaptic contacts were found to be present within the DCX+ cell clusters after detection of the general synaptic marker Syp and the vesicular GABA transporter VGAT (Fig. 8C).

Myelination in neonatal brain

The occurrence of myelin at birth was assessed by Gallyas staining in the corpus callosum of three mammalian species (including Cetartiodactyla and mouse). The analysis showed that myelin sheaths

are already formed in neonatal dolphin, some of them are present in neonatal sheep, but not in mouse (Fig. 9A).

Discussion

The occurrence of DCX+ cells in the white matter of neonatal/young mammals has been reported in several species (Luzzati et al., 2003; Ponti et al., 2006; Inta et al., 2008; Fung et al., 2011; Le Magueresse et al., 2011, 2012; Riccio et al., 2012; Piumatti et al., 2018), including humans (Paredes et al., 2016). Since these cells are prevalently detectable in the perinatal period, and since DCX is commonly thought to be a specific marker for newborn neuroblasts, most investigators consider them as newly generated cortical neurons which can be added during the early postnatal period (Le Magueresse et al., 2011; Paredes et al., 2016). The postnatal migration of neurons (mostly interneurons) coming from the SVZ and passing through the subcortical white matter to finally reach the cerebral cortex, has been shown to occur in rodents (Inta et al., 2008; Le Magueresse et al., 2011,2012; Riccio et al., 2012), humans (Paredes et al., 2016), and, to a lesser extent, rabbits (Luzzati et al., 2003). These results support the view of DCX+ cells in the early postnatal white matter as part of a protracted neurogenic process extending into the young brain and assuring further adjustments of the neural circuits. In parallel, it is more and more evident that certain populations of DCX+ cells can also persist in the postnatal/adult brain as "immature neurons", namely, non-newly generated cells which are born prenatally then continuing to express markers of immaturity for certain periods (Gómez-Climent et al., 2008; Piumatti et al., 2018). Accordingly, DCX as a marker would not be restricted to newlyborn cells but extended to postmitotic neuronal populations supposed to play unknown roles in structural plasticity (Bonfanti and Nacher, 2012; König et al., 2016). Recently, we hypothesized that the occurrence and amount of these immature neurons in mammals could be strictly linked to the animal species, being more abundant in large-brained, long-living mammals with respect to rodents (Bonfanti, 2016; Piumatti et al., 2018). Here, in accord with such hypothesis, we show that large amounts of DCX+ cells form chain-like clusters in the white matter of neonatal and young Cetartiodactyla (dolphins and sheep). Although their morphology is reminiscent of the "chain migration" existing in the rostral migratory stream (Lois et al., 1996; Peretto et al., 1999), they are not the product of postnatal neurogenic activity. Such a conclusion is supported by several converging observations: *i)* analyses conducted with a local cell proliferation marker (Ki-67 antigen) in both animal groups did not reveal any sign of cell proliferation within the clusters; *ii*) most DCX+ cells forming the clusters in sheep are generated prenatally, as revealed by BrdU staining in neonates after

treatment of pregnant ewes three months before birth (injections concentrated in a very short time window which underestimate the number of prenatally-generated cells); *iii*) a negligible neurogenic activity is present in the SVZ-like region of dolphins (Parolisi et al., 2017); *iv*) a continuous gap devoid of DCX+ cells/clusters exists in both species between the SVZ and the area occupied by the clusters (absence of continuity between the neurogenic zone and the DCX+ cells in the white matter). In addition, unlike reports of migrating neurons in mouse and human neonatal brain, the DCX+ cells in the white matter of Cetartiodactyla were never observed to reach the deep cortical layers (summarized in Fig. 10). As shown in a previous report carried out in our own lab on rabbits, if chains of DCX+ cells do connect the SVZ to the cortex this can be easily revealed by serial section analysis (Ponti et al., 2006).

As to the nature of the cells in the clusters, more detailed investigation was carried out in sheep. Most cells were immunopositive for markers of immaturity (DCX and PSA-NCAM) and substantially negative for NeuN (an RNA-binding protein expressed by most postmitotic neurons that start differentiation; Mullen et al., 1992). This pattern confirms the general state of immaturity of the white matter cell clusters, at least in neonates; their expression of "embryonic" N-CAM (PSA-NCAM; Bonfanti, 2006), higher in neonates and lower in young animals (Fig. 8 A1,A2), indicates that the high degree of immaturity detectable at birth progressively declines with time. Accordingly, the clusters contain far less DCX+ cells in young animals in comparison with neonates (as shown by the DAPI-counterstained specimens; Fig 5D). The loss of molecular signs of immaturity, in contrast with the absence of differentiation markers (i.e. NeuN), suggests that the cell clusters do not progress towards a mature neuronal fate. This is further confirmed by the absence of immunoreactivity for markers of both GABAergic interneurons (GAD67) and mature excitatory neurons CAMKII (Varea et al., 2011; Fig 8B), and by substantial lack of synaptic contacts in the clusters (detectable with Syp and VGAT molecular markers, as evident in cerebral cortex controls). Hence, the dorsal white matter cell clusters show feature which are expected in cells which are not fated to further differentiation, rather to disappear, being not detectable even with cresyl violet staining in adults (see schematic summary in Fig. 8C). Whether they slowly die (no signs of picnotic nuclei were detectable at any time) or migrate through white matter is at present unknown.

Taken together, the present results strongly suggest that in Cetartiodactyla the last wave of embryonic neurogenesis would occur through chain migration, then remaining "trapped" within the white matter due to an advanced developmental and maturational stage of the brain (Reidenberg and Laitman, 2002; Garwicz et al., 2009). Unlike most mammals, newborn cetaceans represent an extremely precocial state of development, since they have to swim and surface immediately after birth (Reidenberg and Laitman, 2002; Rauschmann et al., 2006). Accordingly, their brain has attained an

advanced stage of development at birth, as indicated by the average brain weight/adult brain weight (42,5%; Ridgway, 1990; see Table 7) and by several neuroanatomical features (e.g., forebrain neuroanatomy, postnatal genesis of granules in the cerebellar cortex, absence of neurogenesis in the sub-ventricular germinal layer, and perinatal myelination; see Parolisi et al., 2015). Sheep also are prenatal brain developers, their brain weight having reached 50% of adult size at birth (McIntosh et al., 1979; see Table 7). In mouse, humans and rabbits the brain weight at birth is only 10-30% of that reached in adults. Accordingly, the overall stage of neuroanatomical development in Cetartiodactyla is very advanced at birth: around 65% in comparison with 15-35% of mouse, humans and rabbits (References in Table 7). Though nonlinear, the neurodevelopmental schedule seems to be highly conserved and predictable in all eutherian mammals. Precocial ungulates such as sheep, who must be ready to run by birth, have extended gestation and delayed birth to match conserved parameters of brain development (not by selectively advancing the general rate of brain maturation or the specific ability to walk; Finlay and Uchiyama, 2017). Rather than slow the rate of neurogenesis and brain development overall, sheep, appear to delay the onset of initial brain production. Such "adjustment" to neurodevelopment might possibly produce stranding population of cells within the white matter.

One further element considered to be crucial in structural plasticity stabilization during brain maturation is myelin. In precocious animals, myelination is nearly complete at birth, whereas in most mammals it occurs relatively late in postnatal development (Table 7 and Fig. 9). In mice and rats, it starts at birth in the spinal cord but the brain is involved only in postnatal life, so that most of the axons in the corpus callosum are non-myelinated in the neonate (Vincze et al., 2008; Fig 9B). In humans, the peak of myelination occurs much later, during the first year of life, in coincidence with the development of motor abilities and specific cognitive functions (Snaidero and Simons, 2014). On the other hand, as reported previously (Parolisi et al., 2015) and confirmed here (Fig. 9), a high degree of myelination in dolphin, and to a lesser extent in sheep, is detectable at birth (Fig 9A). It is well known that myelin-associated inhibitors lead to restriction of plasticity by promoting stabilization in the adult CNS, also resulting responsible for regeneration failure after lesion (Schwab, 2010; Xu et al., 2015). The early myelination in newborn Cetartiodactyla might contribute to hamper the final migration of the last DCX+ cell clusters. Possibly, in very large brains, the simple physical bulk of even the leading edge of myelinogenesis gets in the way of migration. Interestingly, animals with smaller brains, such as rabbits, seem to display an intermediate situation from the single cell migration of mice/humans and the "trapped-like" clusters of Cetartiodactyla: large chains of newly-generated and proliferating DCX+ cells still reach the cortex at peripuberal stages (Luzzati et al., 2003; Ponti et al., 2006). For some unknown reasons the young rabbit brain tissue seems to be quite permissive to neuroblasts performing chain migration through the white matter (a fact fitting with the striking

neurogenesis still present in the young/adult cerebellum, a region undergoing early stabilization in other mammals; Ponti et al., 2008). Finally, it is worth noting that most clusters of DCX+ cells in both species here investigated lie beneath cortical areas subtending functions particularly important for their early life (somatosensory and motor areas in sheep, including those for tongue and chewing - Borgatti et al., 1956; auditory cortex in dolphins, strictly linked to echolocation - van Kann et al., 2017; Fig. 10B).

The present results support the view that structural/developmental plasticity can highly vary in the brain of different mammals as to its types and topographical distribution. In a previous report (Piumatti et al., 2018), clusters of immature, DCX+ cells are also present within the sheep external capsule. Both cell populations (dorsal white matter and external capsule) are mostly of pallial origin, since they largely express Tbr1 (Fig. 8 A4). Yet, if the two groups of clusters share the embryologic origin, those in the external capsule have been shown to steadily persist in adults (Piumatti et al., 2018). Also, the positive staining for GAD67 previously described for postnatal migrating neuroblasts in mouse and humans (Le Magueresse et al., 2011; Paredes et al., 2016) underlines the high heterogeneity/different nature of the DCX+ cells in the white matter of different animal species. Until recently, this kind of cells was thought to be restricted to the cerebral cortex layer II (Gómez-Climent et al., 2008), yet, they can be more widely distributed in other brain regions, when large-brained mammals are concerned (Piumatti et al., 2018 and this work). Since structural plasticity substantially consists of the retention of immature (embryonic-like) features in the mature, relatively hard-wired brain (Bonfanti, 2006; Peretto et al., 2005; Urbán and Guillemot, 2014), it is quite logic that its extension and location could be linked to the particular CNS developmental history in each species, a feature which is temporally variable in mammals (Garwicz et al., 2009; Workman et al., 2013). Such heterogeneity, in terms of different combinations of neurogenic and/or non-neurogenic processes across mammals, can set some limits for laboratory rodents as prevalent animal models in research as far as structural plasticity involving whole cells/cell populations is concerned (see also Feliciano et al., 2015; Lipp and Bonfanti, 2016; Faykoo-Martinez et al., 2017; Piumatti et al., 2018). Finally, a better knowledge of the neurogenic/plastic events occurring during the early postnatal period can be important in view of their great impact on overall brain function later in life, not only in physiology but also in pathology (Kuhn and Blomgren, 2011). In early postnatal humans, a ventral route, branching off the rostral migratory stream directed to the olfactory bulb, takes young neurons into the medial prefrontal cortex (the so-called medial migratory stream; Sanai et al., 2011). This observation suggests the possibility that migration in large clusters/chains might occur in the last phases of human (prenatal) brain neurogenesis, thus exposing the cells to the risk of "being trapped". Only future, detailed studies carried out on human fetal material would clarify this point.

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Research involving Human Participants and/or Animals: Not applicable

References

Bagley C (1922) Cortical motor mechanism of the sheep brain. Arch Neurol Psych 7: 417-453.

Bonfanti L, Olive S, Poulain DA, Theodosis DT (1992) Mapping of the distribution of polysialylated neural cell adhesion molecule throughout the central nervous system of the adult rat: an immunohistochemical study. Neuroscience 49: 419-436. doi: 10.1016/0306-4522(92)90107-D

Bonfanti L (2006) PSA-NCAM in mammalian structural plasticity and neurogenesis. Prog Neurobiol 80: 129-164. doi: 10.1016/j.pneurobio.2006.08.003

Bonfanti L, Nacher J (2012) New scenarios for neuronal structural plasticity in non-neurogenic brain parenchyma: the case of cortical layer II immature neurons. Prog Neurobiol 98:1-15. doi: 10.1016/j.pneurobio.2012. 05.002

Bonfanti L (2016) Adult neurogenesis 50 years later: Limits and opportunities in mammals. Front Neurosci 10:44. doi: 10.3389/fnins.2016.00044

Bonner JT (1980) The evolution of culture in animals. Princeton University Press, Princeton, New Jersey.

Borgatti G (1956) Centri nervosi e sistema nervoso centrale. In: Borgatti G, Martini E, Rowinski P, Usuelli F, Fisiologia degli animali domestici. Tinarelli, Bologna, p. 1382.

Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG (2003) Transient expression of doublecortin during adult neurogenesis. J Comp Neurol 467:1-10. doi: 10.1002/cne.10874

Brus M, Keller M, Lévy F (2013a) Temporal features of adult neurogenesis: differences and similarities across mammalian species. Front Neurosci 7:135-144. doi: 10.3389/fnins.2013.00135

Brus M, Meurisse M, Gheusi G, Keller M, Lledo P, Levy F (2013b) Dynamics of olfactory and hippocampal neurogenesis in adult sheep. J Comp Neurol 521: 169-188. doi: 10.1002/cne.23169

Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Edwards RH, Storm-Mathisen J (1998) The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. J Neurosci 18: 9733-9750.

Cozzi B, Huggenberger S, Oelschläger HHA (2017) The anatomy of dolphins. Insights into body structure and function. Chapter 6: Brain, Spinal Cord, and Cranial Nerves. Academic Press, London, pp 191-285.

Dekaban AS, Sadowsky D (1978) Changes in brain weights during the span of human life: Relation of brain weigths to body heigths and body weigths. Ann Neurol 4: 345-356. doi: 10.1002/ana.410040410

Faykoo-Martinez M, Toor I and Holmes MM (2017) Solving the Neurogenesis Puzzle: Looking for Pieces Outside the Traditional Box. Front Neurosci 11:505. doi: 10.3389/fnins.2017.00505. doi: 10.3389/fnins.2017.00505

Feliciano DM, Bordey A, Bonfanti L (2015) Noncanonical Sites of Adult Neurogenesis in the Mammalian Brain. Cold Spring Harb Perspect Biol 7:a018846. doi: 10.1101/cshperspect.a018846

Finlay BL, Uchiyama R (2017) The timing of brain maturation, early experience, and the human social niche. In: Kaas, J (ed.), Evolution of Nervous Systems 2e. vol. 3, pp. 123-148. Oxford: Elsevier.

Fung SJ, Joshi D, Allen KM, Sivagnanasundaram S, Rothmond DA, Saunders R, Noble PL, Webster MJ, Shannon Weickert CS (2011) Developmental patterns of doublecortin expression and white matter neuron density in the postnatal primate prefrontal cortex and schizophrenia. PLoS ONE 6(9): e25194. doi: 10.1371/journal.pone.0025194

Gallyas F (1979) Silver staining of myelin by means of physical development. Neurol Res 1, 203-209.

Garwicz M, Christensson M, Psounib E (2009) A unifying model for timing of walking onset in humans and other mammals. Proc Natl Acad Sci USA 106: 21889-21893. doi: 10.1073/pnas.0905777106

Gatesy J, Milinkovitch M, Waddell V, Stanhope M (1999) Stability of cladistic relationships between Cetacea and higher-level Artiodactyl taxa. Syst Biol 48: 6-20.

Geraci JR, Lounsbury VJ (2005) Marine Mammals Ashore: A Field Guide for Strandings, Second Edition. National Aquarium in Baltimore, Baltimore, MD.

Gleeson JG, Lin PT, Flanagan LA, Walsh CA (1999) Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23:257–271. doi: 10.1016/S0896-6273(00)80778-3

Gómez-Climent MA, Castillo-Gómez E, Varea E, Guirado R, Blasco-Ibáñez JM, Crespo C, Martínez-Guijarro FJ, Nácher J (2008) A population of prenatally generated cells in the rat paleocortex maintains an immature neuronal phenotype into adulthood. Cereb Cortex 18:2229-2240. doi: 10.1093/cercor/bhm255

Graur D, Higgins DG (1994) Molecular evidence for the inclusion of cetaceans within the order Artiodactyla. Mol Biol Evol 11: 357-364.

Hahn ME, Jensen C, Dudek DC (1979) Development and evolution of brain size: Behavioral implications. Academic Press, New York, USA.

Inta D, Alfonso J, von Engelhardt J, Kreuzberg MM, Meyer AH, van Hooft JA, Monyer H (2008) Neurogenesis and widespread forebrain migration of distinct GABAergic neurons from the postnatal subventricular zone. Proc Natl Acad Sci USA 105:20994-20999. doi: 10.1073/pnas.0807059105

Kazu RS, Maldonado J, Mota B, Manger PR, Herculano-Houzel S (2014) Cellular scaling rules for the brain of Artiodactyla include a highly folded cortex with few neurons. Front Neuroanat 8:128. doi: 10.3389/fnana.2014.00128

Kee N, Sivalingam S, Boonstra R, Wojtowicz JM (2002) The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods 115, 97-105.

Kern, A., Siebert, U., Cozzi, B., Hof, P.R., Oelschlager, H.H.A., 2011. Stereology of the neocortex in Odontocetes: qualitative, quantitative, and functional implications. Brain Behav Evol 77, 79–90. doi: 10.1159/000323674

König R, Benedetti B, Rotheneichner P, O′Sullivan A, Kreutzer C, Belles M, Nacher J, Weiger TM, Aigner L, Couillard-Després S (2016) Distribution and fate of DCX/PSA-NCAM expressing cells in the adult mammalian cortex: A local reservoir for adult cortical neuroplasticity? Front Biol 11: 193-213. doi: 10.1007/s11515-016-1403-5

Kuhn GH, Blomgren K (2011) Developmental dysregulation of adult neurogenesis. Eur J Neurosci 33: 1115-1122. doi: 10.1111/j.1460-9568.2011.07610.x

Le Magueresse C, Alfonso A, Khodosevich K, Arroyo Martin AA, Bark C, Monyer H (2011) 'Small axonless neurons': postnatally generated neocortical interneurons with delayed functional maturation. J Neurosci 31:16731-16747. doi: 10.1523/JNEUROSCI.4273-11.2011

Le Magueresse C, Alfonso J, Bark C, Eliava M, Khrulev S, Monyer H (2012) Subventricular zone-derived neuroblasts use vasculature as a scaffold to migrate radially to the cortex in neonatal mice Cereb Cortex 22:2285-2296. doi: 10.1093/cercor/bhr302

Lipp HP, Bonfanti L (2016) Adult neurogenesis in mammals: variations and confusions. Brain Behav Evol 87:205-221. doi: 10.1159/000446905

Lois C, Garcìa-Verdugo JM, Alvarez-Buylla A, (1996) Chain migration of neuronal precursors. Science 271: 978-981. doi: 10.1126/science.271.5251.978

Luzzati, F., Peretto, P., Aimar, P., Ponti, G., Fasolo, A, Bonfanti, L (2003) Glia-independent chains of neuroblasts through the subcortical parenchyma of the adult rabbit brain. Proc. Natl Acad. Sci. USA, 100, 13036-13041. doi: 10.1073/pnas.1735482100

Luzzati F, Bonfanti L, Fasolo A, Peretto P (2009) DCX and PSA-NCAM expression identifies a population of neurons preferentially distributed in associative areas of different pallial derivatives and vertebrate species. Cereb Cortex 19:1028-41. doi: 10.1093/cercor/bhn145

McIntosh GH, Baghurst I, Potter BJ, Hetzel BS (1979) Foetal brain development in the sheep. Neuropath Appl Neurobiol 5,103-114. doi: 10.1111/j.1365-2990.1979.tb00664.x

Mullen RJ, Buck CR, Smith AM (1992) NeuN, a neuronal specific nuclear protein in vertebrates. Development 116: 201-211.

Murphy WJ, Pevzner PA, O'Brien SJ (2004) Mammalian phylogenomics comes of age. Trends Genet 20: 631-639. doi: 10.1016/j.tig.2004.09.005

Nacher J, Crespo C, McEwen BS (2001) Doublecortin expression in the adult rat telencephalon. European J Neurosci 14: 629-644. doi: 10.1046/j.0953-816x.2001.01683.x

Paredes, MF, James, D, Gil-Perotin, S, Kim, H, Cotter, JA, Ng, C, Sandoval, K, Rowitch, DH, Xu, D, McQuillen, PS, Garcia-Verdugo, J-M, Huang, EJ, Alvarez-Buylla, A (2016) Extensive migration of young neurons into the infant human frontal lobe. Science 354 (6308). doi: 10.1126/science.aaf7073

Parolisi R, Peruffo A, Messina S, Panin M, Montelli S, Giurisato M, Cozzi B, Bonfanti L (2015) Forebrain neuroanatomy of the neonatal and juvenile dolphin (*T. truncatus* and *S. coeruloalba*). Front Neuroanat. 9: 140. doi: 10.3389/fnana.2015.00140

Parolisi R, Cozzi B, Bonfanti L (2017) Non-neurogenic SVZ-like niche in dolphins, mammals devoid of olfaction. Brain Struct Funct 222: 2625-2639. doi: 10.1007/s00429-016-1361-3

Peretto P, Merighi A, Fasolo A, Bonfanti L (1999) The subependymal layer in rodents: a site of structural plasticity and cell migration in the adult mammalian brain. Brain Res Bull 49: 221-243. doi: 10.1016/S0361-9230(99)00037-4

Peretto P, Giachino C, Aimar P, Fasolo A, Bonfanti L (2005) Chain formation and glial tube assembly in the shift from neonatal to adult subventricular zone of the rodent forebrain. J Comp Neurol 487: 407-427. doi: 10.1002/cne.20576

Piumatti M, Palazzo O, La Rosa C, Crociara P, Parolisi R, Luzzati F, Lévy F, Bonfanti L (2018) Non-newly generated, "immature" neurons in the sheep brain are not restricted to cerebral cortex. J Neurosci E-pub. doi: 10.1523/JNEUROSCI.1781-17.2017

Ponti G, Aimar P, Bonfanti L (2006) Cellular composition and cytoarchitecture of the rabbit subventricular zone (SVZ) and its extensions in the forebrain. J Comp Neurol 498: 491-507. doi: 10.1002/cne.21043

Ponti G, Peretto P, Bonfanti L (2008) Genesis of neuronal and glial progenitors in the cerebellar cortex of peripuberal and adult rabbits. PLoS ONE 3:e2366. doi: 10.1371/journal.pone.0002366

Poth C, Fung C, Gunturkun O, Ridgway SH, Oelschlager HHA (2005) Neuron numbers in sensory cortices of five delphinids compared to a physeterid, the pygmy sperm whale. Brain Res Bull 66: 357-360. doi: 10.1016/j.brainresbull.2005.02.001

Price SA, Bininda-Emonds OR, Gittleman JL (2005) A complete phylogeny of the whales, dolphins and even-toed hoofed mammals (Cetartiodactyla). Biol Rev Camb Philos Soc 80: 445-473.

Puelles L, Kuwana E, Puelles E, Bulfone A, Shimamura K, Keleher J, Smiga S, Rubenstein JL (2000) Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes *Dlx*-2, Emx-1, Nkx-2.1, *Pax*-6, and *Tbr*-1. J Comp Neurol 424:409-438.

Rauschmann MA, Huggenberger S, Kossatz LS, Oelschlager HHA (2006) Head morphology in perinatal dolphins: A window into phylogeny and ontogeny. J Morphol 267: 1295-1315. doi: 10.1002/jmor.10477

Reidenberg JS, Laitman JT (2002) Prenatal development in cetaceans. In: Perrin WF, Wursig B, Thewissen JGM, editors. Encyclopedia of Marine Mammals. San Diego: Academic Press, pp 998-1007.

Riccio O, Murthy S, Szabo G, Vutskits L, Kiss JZ, Vitalis T, Lebrand C, Dayer AG (2012) New pool of cortical interneuron precursors in the early postnatal dorsal white matter. Cereb Cortex 22: 86-98. doi: 10.1093/cercor/bhr086

Ridgway SH (1990) The central nervous system of the bottlenose dolphin. In: The Bottlenose Dolphin, eds S. Leatherwood and R.R. Reeves, Waltham MA, Academic Press, 69-97.

Sanai N, Nguyen T, Ihrie RA, Mirzadeh Z, Tsai HH, Wong M, Gupta N, Berger MS, Huang E, Garcia-Verdugo JM, Rowitch DH, Alvarez-Buylla A (2011) Corridors of migrating neurons in the human brain and their decline during infancy. Nature 478: 382-386. doi: 10.1038/nature10487

Schwab ME (2010) Functions of Nogo proteins and their receptors in the nervous system. Nat Rev Neurosci 11: 799-811. doi: 10.1038/nrn2936

Snaidero N, Simons M (2014) Myelination at a glance. J Cell Sci 127: 2999-3004. doi: 10.1242/jcs.151043

Taylor CR, Shi SR, Chaiwun B, Young L, Imam SA, Cote RJ (1994) Strategies forimproving the immunohistochemical staining of various intranuclear prognostic markers in formalin-paraffin sections: androgen receptor, estrogen receptor, progesterone receptor, p53 protein, proliferating cell nuclear antigen, and Ki-67 antigen revealed by antigen retrieval techniques. Hum Pathol 25: 263-270.

Urbán N, Guillemot F (2014) Neurogenesis in the embryonic and adult brain: same regulators, different roles. Front Cell Neurosci 8: 396. doi: 10.3389/fncel.2014.00396

van Kann E, Cozzi B, Hof PR, Oelschläger HHA (2017) Qualitative and quantitative analysis of primary neocortical areas in selected mammals. Brain Behav Evol E-pub doi: 10.1159/000477431.

Varea E, Belles M, Vidueira S, Blasco-Ibáñez JM, Crespo C, Pastor ÁM and Nacher J (2011) PSA-NCAM is expressed in immature, but not recently generated, neurons in the adult cat cerebral cortex layer II. Front Neurosci 5:17. doi:10.3389/fnins.2011.00017

Vincze A, Mazlo M, Seress L, Komoly S, Abraham H (2008) A correlative light and electron microscopic study of postnatal myelination in the murine corpus callosum. Int J Dev Neurosci 26: 575-584. doi: 10.1016/j.ijdevneu.2008.05.003

Wiedenmann B, Franke WW (1985) Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of presynaptic vesicles. Cell 41: 1017-1028.

Workman AD, Charvet CJ, Clancy B, Darlington RB, Finlay BL (2013) Modeling transformations of neurodevelopmental sequences across mammalian species. J Neurosci 33: 7368-7383. doi: 10.1523/JNEUROSCI.5746-12.2013

Xu CJ, Wang JL, Jin WL (2015) The neural stem cell microenvironment: Focusing on axon guidance molecules and myelin-associated factors. J Mol Neurosci 56: 887-897. doi: 10.1007/s12031-015-0538-1

Zamenhof S, van Marthens E (1976) Neonatal and adult brain parameters in mice selected for adult brain weight. Dev Psychobiol 9: 587-593. doi: 10.1002/dev.420090612

Figure legends

Figure 1. A, Phylogenetic relationships of Cetartiodactyla including Cetacea and Artiodactyla (adapted from Price et al., 2005; in blue, families considered in this study). B, Animal species analysed. C, Comparable anatomical levels (L1-L4, anterior to posterior) in dolphin and sheep brain. Dark grey, grey matter; light gray, white matter.

Figure 2. Clusters of immature cells in the subcortical white matter of dolphin (A) and sheep (B), visualized by histology and confocal microscopy immunodetection. A1, B1, cresyl violet staining revealing tightly-packed clusters of cells (black arrows); A2, A3, B2, immunocytochemical detection of DCX (A4-A6, B3, B4, in combination with glial markers). Mtd, maximum transversal diameter. Scale bars: 50 μm.

Figure 3. Topographical distribution and amount of the DCX+ cell clusters in the neonatal dolphin. A, The red dots indicate the position of clusters in the white matter at different levels of the three animals analyzed (ID: 186, 343, 145); slice blocks analyzed are delineated by black lines. B, An example (based on three brain sections) of the method used to define the average areas occupied by the clusters. C, Regions of the white matter hosting the clusters (pink areas) at four representative brain levels, and quantification of the DCX+ cell clusters expressed as density/area.

Figure 4. Topographical distribution and amount of the DCX+ cell clusters in the neonatal sheep. A, The red dots indicate the position of clusters in the white matter at different levels of the three animals analysed (ID: 372, 207, 373). B, Regions of the white matter hosting the clusters (pink areas) at four representative brain levels. C, Quantification of the DCX+ cell clusters expressed as density/area (cells/mm^2).

Figure 5. DCX+ cell clusters in the white matter of young Cetartiodactyla. A-D, Occurrence of DCX+ cell clusters in the young sheep (animals ID: 915, 920, 990). A, Topographical distribution (red dots); B, regions of the white matter hosting the clusters (pink areas) at four representative brain levels; C, quantification of the DCX+ cell clusters expressed as cluster density/area (showing no significant differences if compared with that observed in the newborn; see Fig. 4). D, Photographs of the cell clusters in newborn (N) and young (Y) sheep; confocal images show that only some cells are DCX+ in young with respect to newborn. E-G, Qualitative analysis in a specimen of young dolphin (*S. coeruloalba*; animal ID: 320); E, clusters of DCX+ cells (arrows), frequently associated to blood

vessels (asterisks) revealed by Cresyl violet staining; F, topographical distribution (blue dots, referring to a single specimen of young dolphin) at the four brain levels; G, Summary concerning the gap (distance in µm) from periventricular SVZ-lr (green) to closest clusters (average values considering all brain levels in a single specimen); light blue, area occupied by DCX+ cells/clusters (see also Fig. 7). Scale bars: 50 µm (light microscopy); 25 µm (confocal microscopy).

Figure 6. Proliferative activity detected with cell proliferation markers Ki-67 (A, dolphin and sheep) and BrdU (sheep, B) in the white matter areas hosting DCX+ cell clusters in neonatal Cetartiodactyla. In both species, detection of Ki-67 antigen revealed rare proliferative cells (arrows) in the tissue among the clusters (Cl); quantification in Table 5), never in association with any of the DCX+ cells. By contrast, in neonatal sheep whose mothers have been injected with BrdU many DCX+ cells are double labeled (B, yellow arrows). Scale bars: 25 µm.

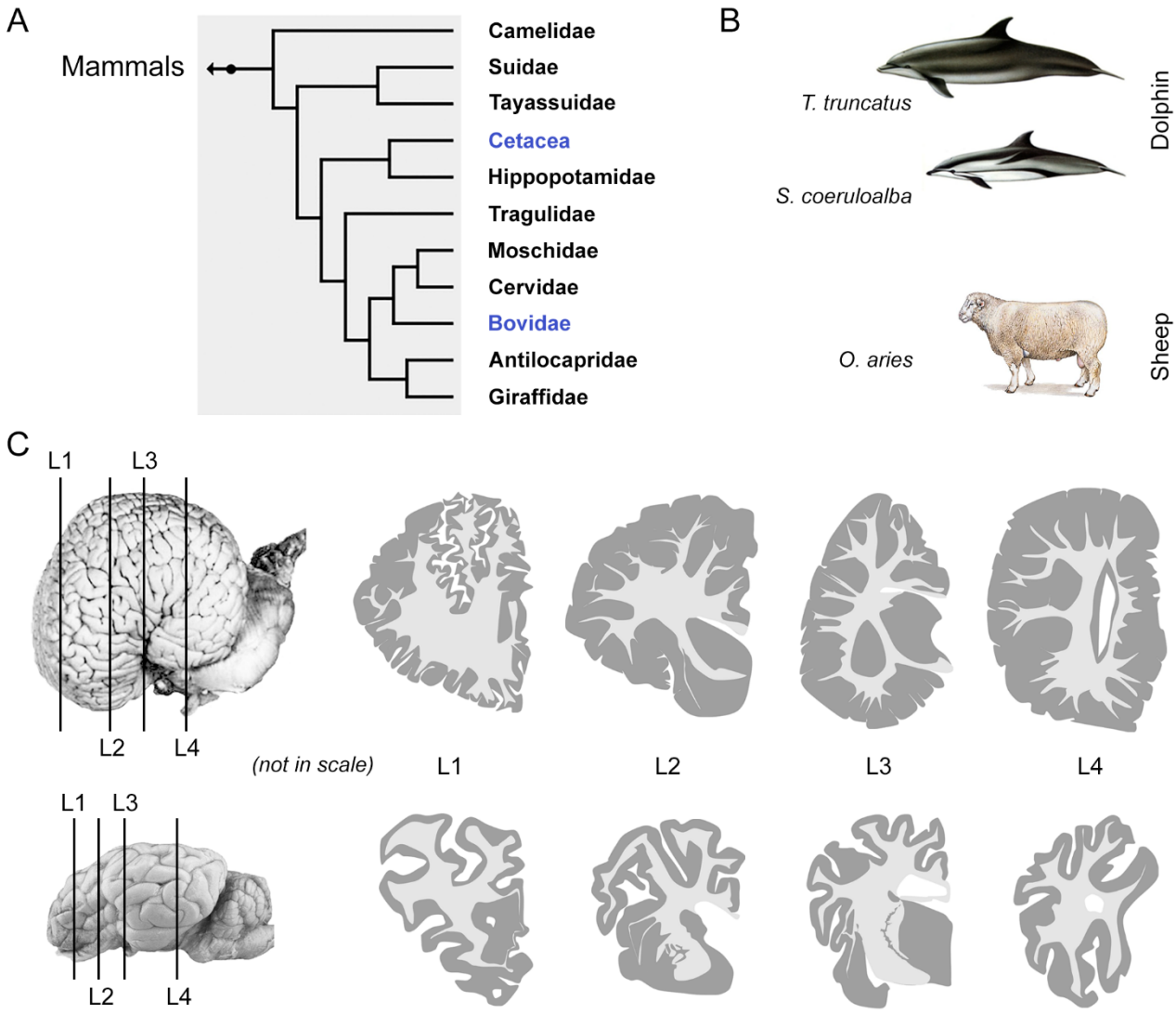
Fig. 7. The DCX+ cell clusters are confined within the white matter. A, A continuous gap always exists between the SVZ region and the closest DCX+ clusters in both dolphin and sheep brain (data provided at the single brain levels). A', Summary of gap distances (µm) with average values considering all brain levels; in pink, area occupied by DCX+ cells/clusters. B, The DCX+ cells/clusters never enter the cortical layers. Only scattered cells are occasionally detectable close to the white/grey matter limit (inset), yet always on the white matter side. No DCX+ cells are present in the deep cortical layers; on the other side, close to the cortical surface, the layer II hosts the typical DCX+ immature neurons, as previously described in most mammals (Gómez-Climent et al., 2008; Bonfanti and Nacher, 2012). C, Schematic summary depicting the distribution of DCX+ cells/clusters always confined within the subcortical white matter. Scale bar: 50 µm.

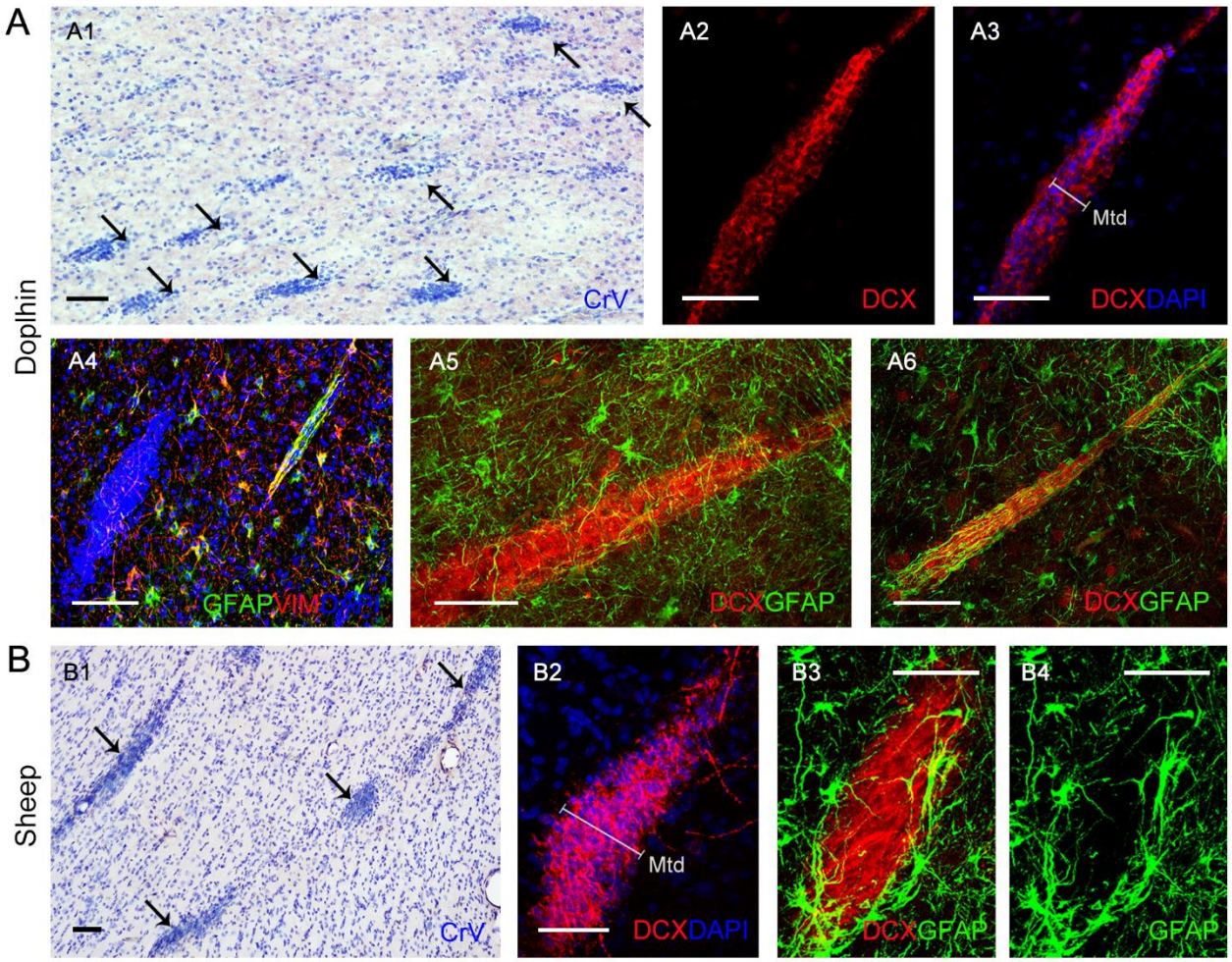
Figure 8. Detection of markers related to maturity/immaturity, embryonic origin, phenotype, synaptic contacts in the sheep white matter cell clusters. A1,A2, Double staining with the marker of immaturity PSA-NCAM (PSA) reveals virtually all DCX+ cells within the clusters in neonatal animals (N) but only a few dots are detectable on some cells in youngs (Y). A3, No DCX+ cells in the clusters are immunopositive for the mature neuron marker NeuN. A4, Most, if not all of the cells express the transcription factor Tbr1, specifically expressed by pallium-derived principal neurons. B, The DCX+ cell clusters do not express markers for mature excitatory neurons (CAMKII, B2) or interneurons (GAD67, not shown; B1,B3, internal positive controls for GAD67 and CAMKII in the cerebral cortex, Cx). C, Molecular markers for synaptic contacts (C1, VGAT, marker for GABAergic terminals; C2, Syp, general marker for synapses) are negligible within the white matter cell clusters.

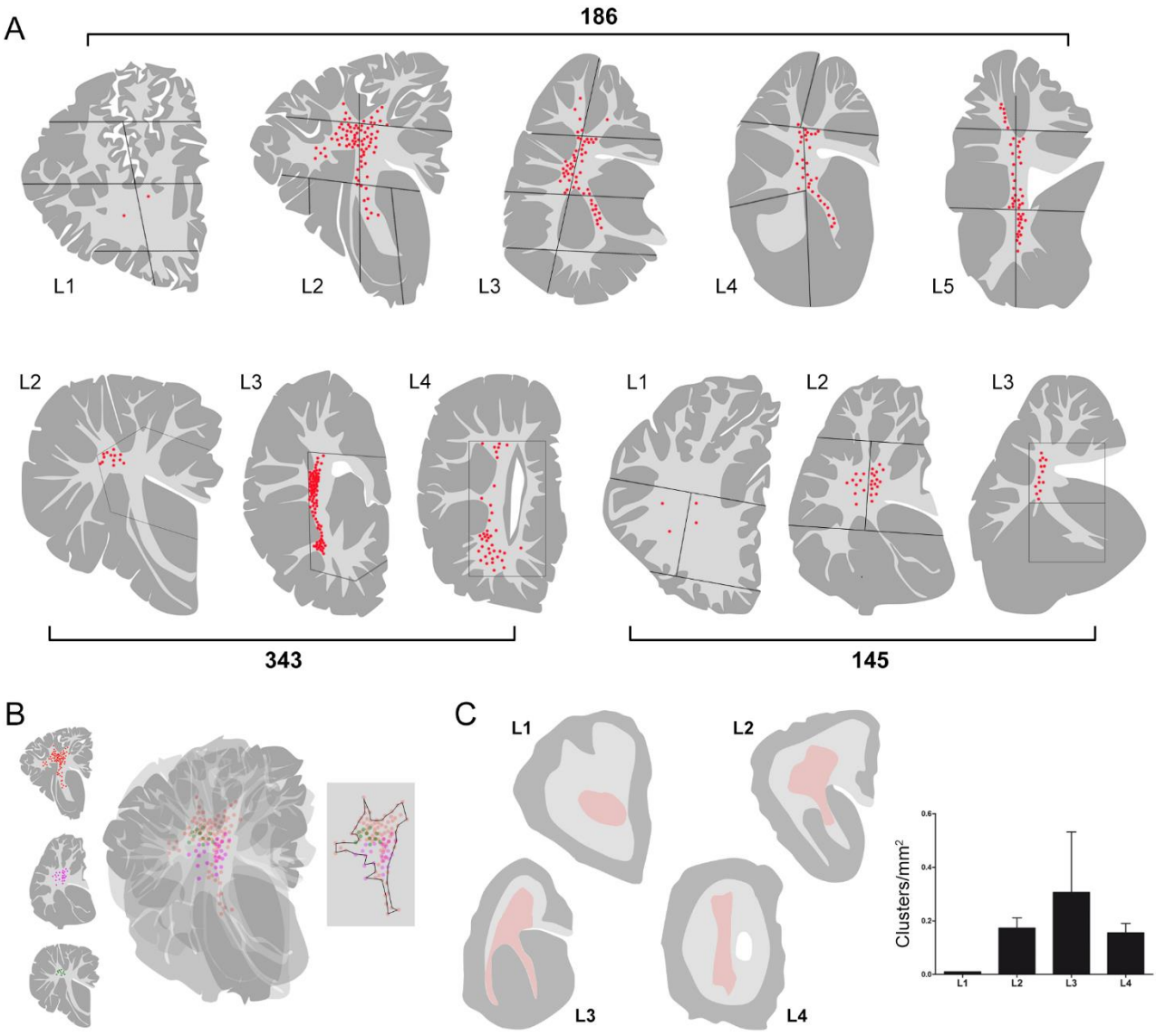
On the right, controls in the cerebral cortex (Cx); C1 and C2 top, sheep; C2 bottom, mouse. Scale bars: 25 µm; C2, right top and bottom, 10 µm.

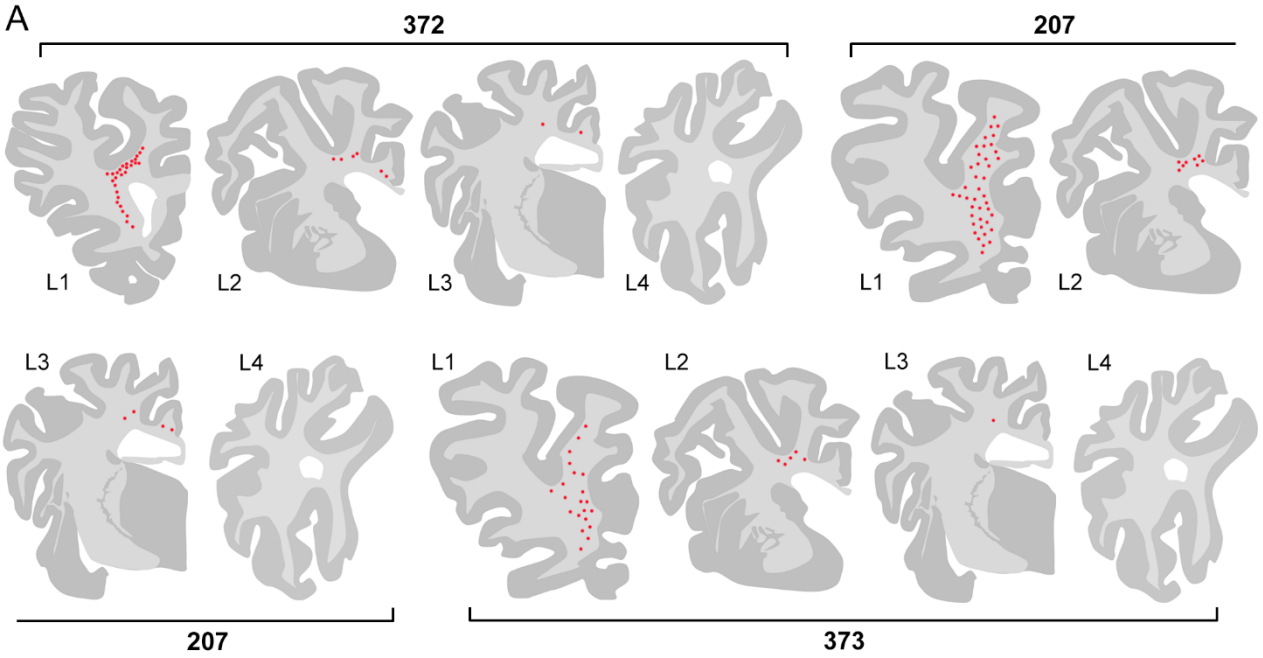
Figure 9. A, Myelination already occurred at birth in the corpus callosum of both dolphin and sheep brain but not mouse (Gallyas staining). FM, fully myelinated; PM, partially myelinated; Un, unmyelinated. Scale bars: 25 µm.

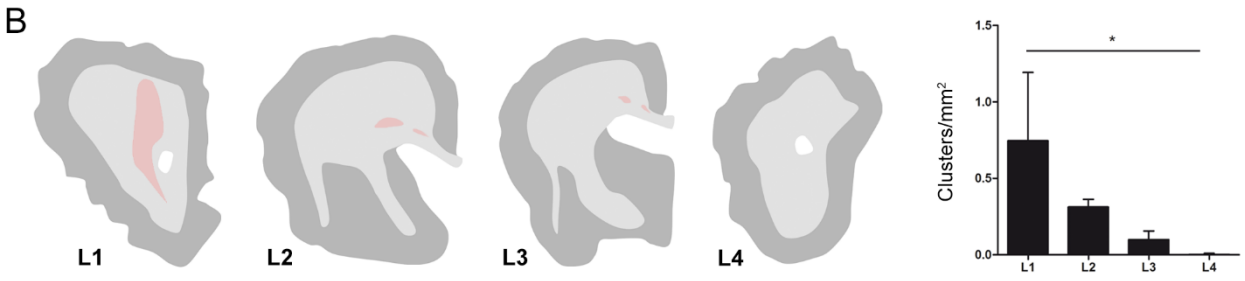
Figure 10. Functional and comparative aspects of DCX+ cell clusters in Cetartiodactyla. A, Schematic synthesis of topographical distribution and amount of the DCX+ cell clusters in the white matter of the dolphin and sheep brains. B, Functional areas of neocortex of sheep and dolphin: adapted from Bagley, 1922, Borgatti et al., 1956 (1), Cozzi et al., 2017 (2). C, Different distribution, orientation and general organization of immature, DCX+ cells in the early postnatal brain of Cetartiodactyla Vs. rodents, humans and rabbits.

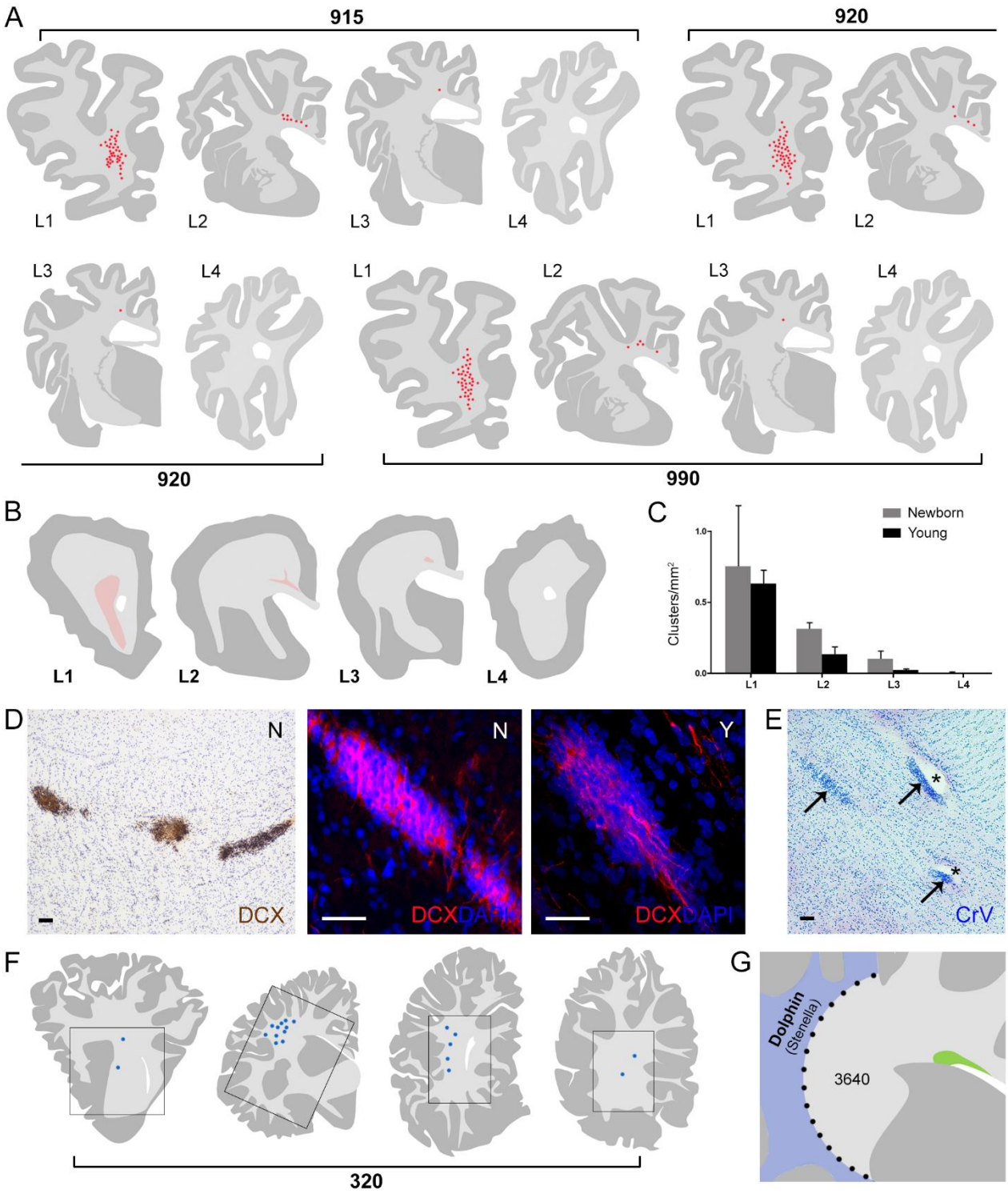

A
Mammals
Camelidae
Suidae
Tayassuidae
Cetacea
Hippopotamidae
Tragulidae
Moschidae
Cervidae
Bovidae
Antilocapridae
Giraffidae
B
T. truncatus
Dolphin
S. coeruloalba
O. aries
Sheep
C
L1
L3
L2
L4
(not in scale)
L1
L2
L3
L4
L1
L3
L2
L4

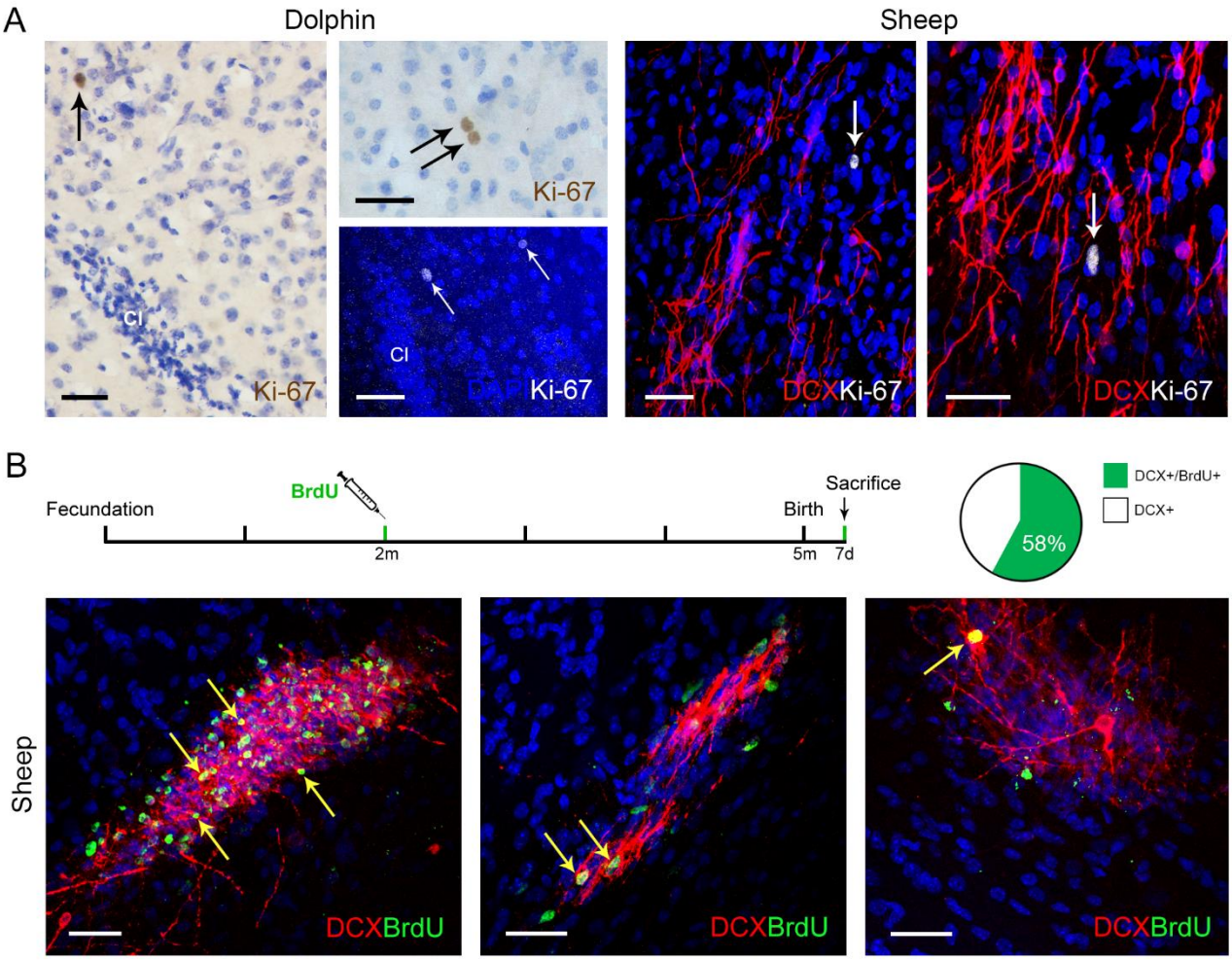

A
A1
A2
A3
Doplhin
CrV
DCX
Mtd
DCXDAPI
A4
A5
A6
GFAPVIMDAPI
DCXGFAP
DCXGFAP
B
B1
B2
B3
B4
Sheep
CrV
Mtd
DCXDAPI
DCXGFAP
GFAP

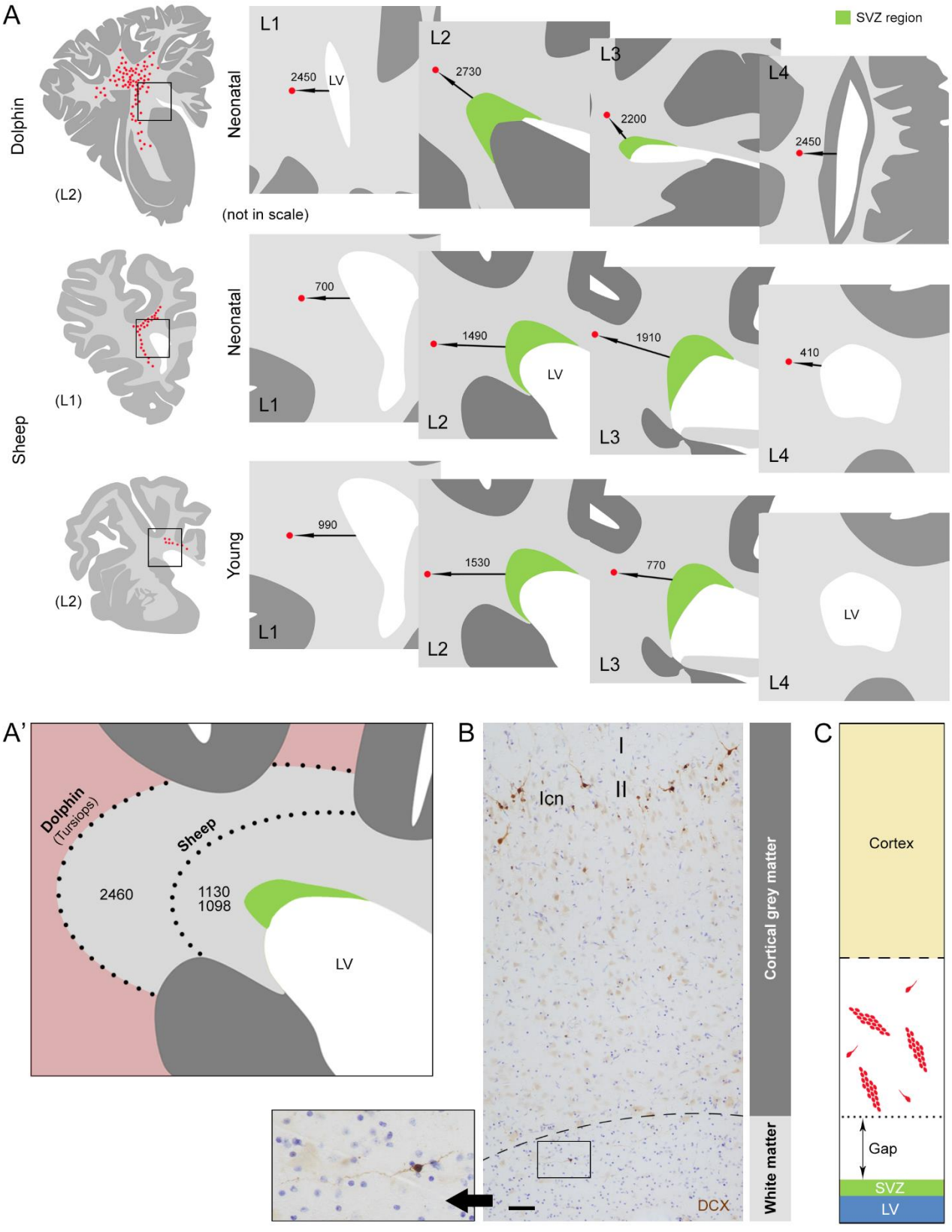

A
186
L1
L2
L3
L4
L5
L2
L3
L4
L1
L2
L3
343
145
B
C
L1
L2
L3
L4
Clusters/mm²
0.6
0.4
0.2
0.0
L1
L2
L3
L4

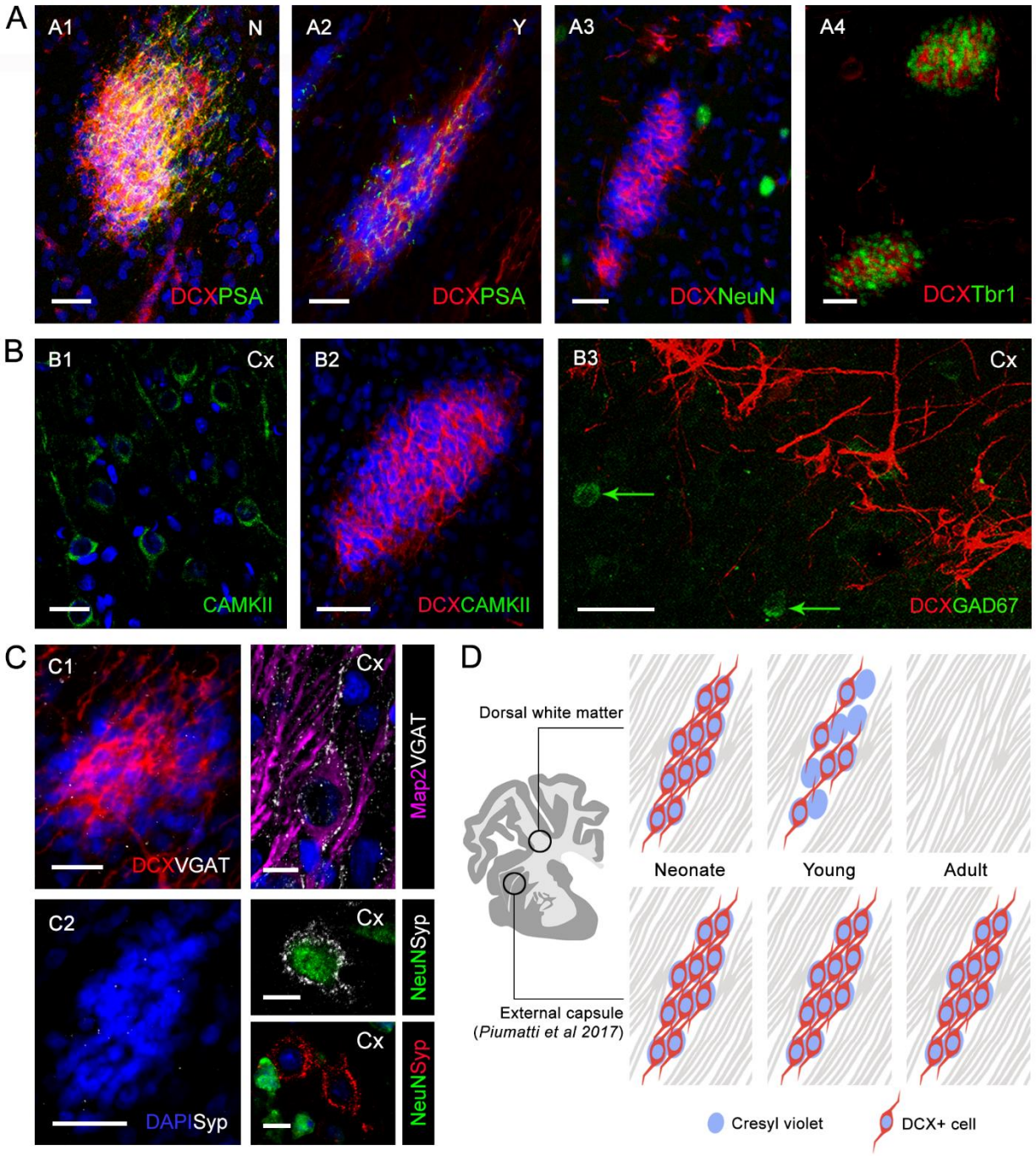

A
372
207
L1
L2
L3
L4
L1
L2
L3
L4
L1
L2
L3
L4
207
373

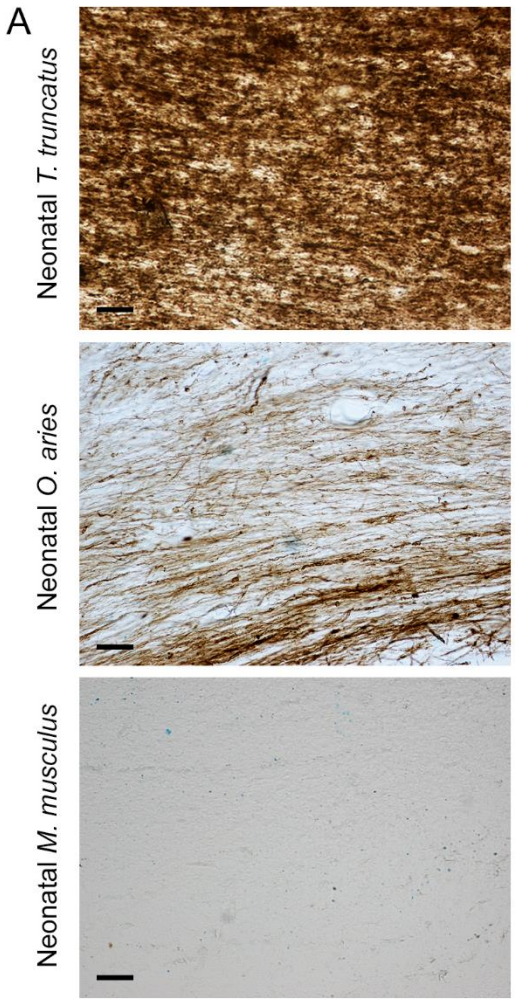

B
L1
L2
L3
L4
Clusters/mm2
1.5
1.0
0.5
0.0
*
L1
L2
L3
L4

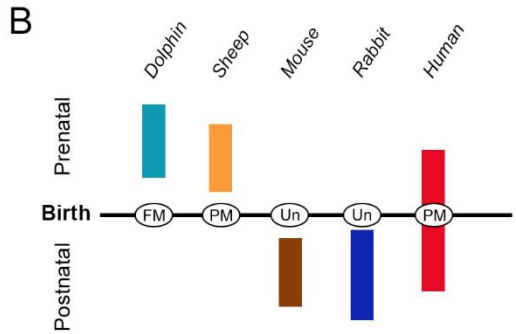

A
915
920
L1
L2
L3
L4
L1
L2
L3
L4
L3
L4
L1
L2
L3
L4
920
990
B
L1
L2
L3
L4
C
Newborn
Young
Clusters/mm²
1.0
0.5
0.0
L1
L2
L3
L4
D
N
DCX
N
DCXDAPI
Y
DCXDAPI
E
CrV
F
320
G
Dolphin
(Stenella)
3640


A
Dolphin
Sheep
Ki-67
Ki-67
CI
CI
DAPI Ki-67
DCXKi-67
DCXKi-67
B
Fecundation
BrdU
2m
Birth
Sacrifice
5m
7d
58%
DCX+/BrdU+
DCX+
Sheep
DCXBrdU
DCXBrdU
DCXBrdU


A
SVZ region
Dolphin
(L2)
Neonatal
L1
2450
LV
L2
2730
L3
2200
L4
2450
(not in scale)
Sheep
(L1)
Neonatal
700
1490
LV
1910
410
(L2)
Young
990
1530
770
LV
A'
Dolphin
(Tursiops)
Sheep
2460
1130
1098
LV
B
I
II
Icn
Cortical grey matter
White matter
DCX
C
Cortex
Gap
SVZ
LV


A
A1
N
DCXPSA
A2
Y
DCXPSA
A3
DCXNeuN
A4
DCXTbr1
B
B1
Cx
CAMKII
B2
DCXCAMKII
B3
Cx
DCXGAD67
C
C1
DCXVGAT
Cx
Map2VGAT
C2
DAPISyp
Cx
NeuNSyp
Cx
NeuNSyp
D
Dorsal white matter
External capsule
(Piumatti et al 2017)
Neonate
Young
Adult
Cresyl violet
DCX+ cell


A
Neonatal T. truncatus
Neonatal O. aries
Neonatal M. musculus


B
Dolphin
Sheep
Mouse
Rabbit
Human
Prenatal
Birth
Postnatal
FM
PM
Un
Un
PM

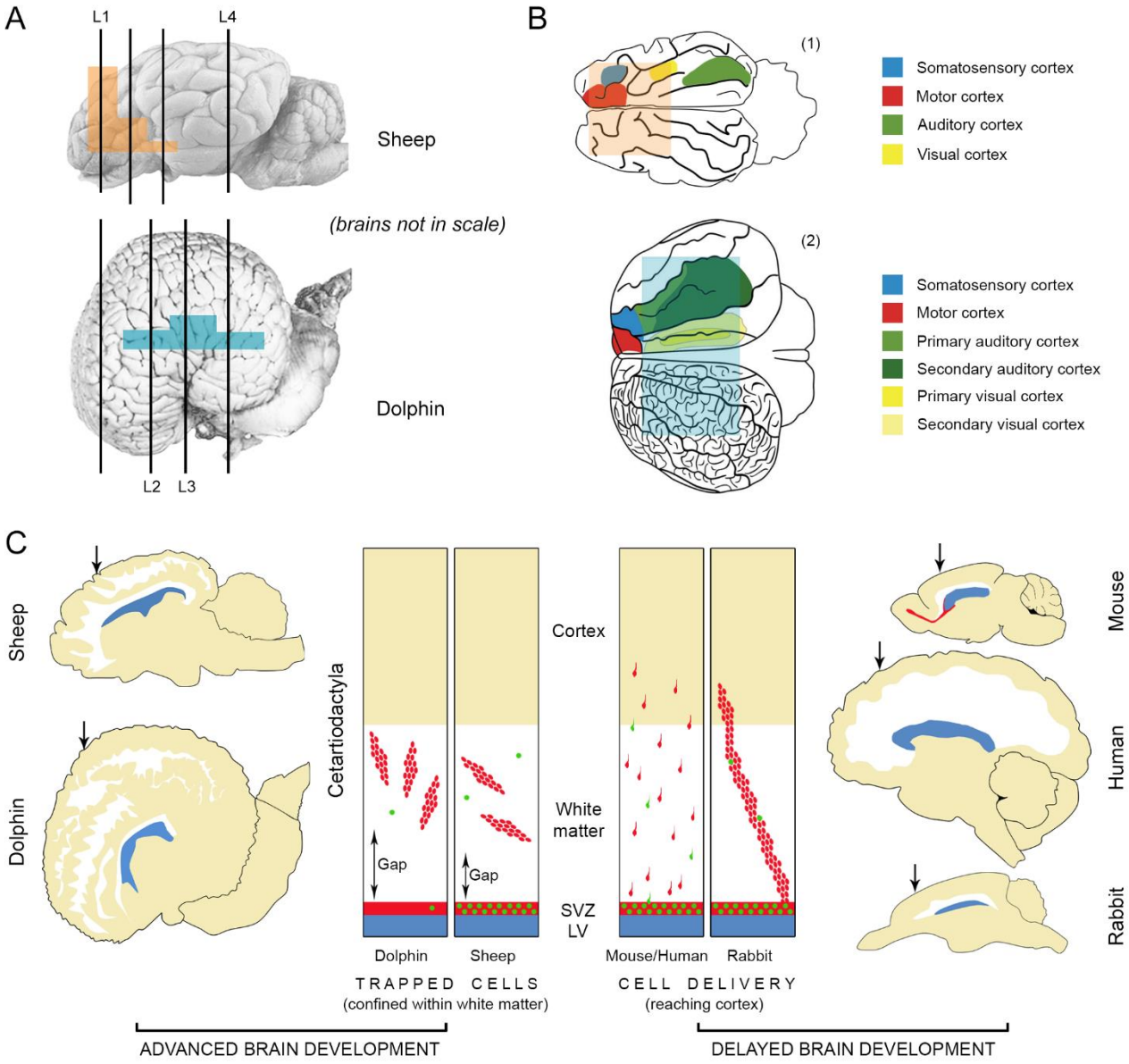

A
L1
L4
Sheep
(brains not in scale)
Dolphin
L2
L3
B
(1)
Somatosensory cortex
Motor cortex
Auditory cortex
Visual cortex
(2)
Somatosensory cortex
Motor cortex
Primary auditory cortex
Secondary auditory cortex
Primary visual cortex
Secondary visual cortex
C
Sheep
Dolphin
Cetartiodactyla
Gap
Gap
Dolphin
Sheep
TRAPPED CELLS
(confined within white matter)
ADVANCED BRAIN DEVELOPMENT
Cortex
White matter
SVZ
LV
Mouse/Human
Rabbit
CELL DELIVERY
(reaching cortex)
DELAYED BRAIN DEVELOPMENT
Mouse
Human
Rabbit

Table 1. Animals used in this study

Species	ID	Sex	Age	BrdU treatment
T. truncatus	186	F	9 days (neonatal) [2]	-
	145	M	7 days (neonatal) [2]	
	343	F	1 day (neonatal) [2]	
	192	F	Adult [2]	
	196	M	Adult [2]	
	319	M	Adult [3]	
S. coeruleoalba	320	F	Young (3-6 months) [2]	
O. aries	207	F	Neonatal (7 days)	Survival: 97 days (injection: 90 days pre-partum)
	372	F		
	373	F		
	208	M		
	209	M		
	370	M		
	915	F	Young (4 months)	-
	920			
	990			
	107		Adult (2 years)	Survival: 120 days
	112			
	338			
	054			
	322			
	447			Survival: 60 days
	464			
	423			Survival: 30 days
	266			

In square brackets, conservation code.

Table 2. Antibodies used in this study

nf, not feasible; np, not performed

Antigen	Host	Source	Dilution	Sheep	Dolphin
DCX	goat	Santa Cruz	1:700-2000	+	-
DCX	rabbit	Abcam	1:500-1800	+	+
Ki-67	mouse	BD Pharmigen	1:1000	+	-
Ki-67	rabbit	Leica-Novocastra	1:600-1000	+	+
BrdU	rat	AbD Serotec	1:300	+	nf
GFAP	rabbit	Dako	1:2000	+	+
Vim	mouse	Exbio	1:800	+	+
PSA-NCAM	mouse	Millipore	1:1400	+	-
GAD67	mouse	Millipore	1:500	+	-
Tbr1	rabbit	Chemicon	1:1000	+	-
NeuN	mouse	Millipore	1:1000	+	-
CAMKII	mouse	Abcam	1:500	+	np
Syp	goat	Santa Cruz	1:500	+	np
VGAT	guinea pig	Synaptic System	1:700	+	np
Map2	goat	Santa Cruz	1:1000	+	np

Species	Age	Level	Cell cluster maximum transversal diameter (µm)	Average (µm)
T.truncatus	Neonatal	1	22.8 ± 5.2	23.7 ± 5
		2	31.2 ± 13.9	
		3	19.6 ± 3	
		4	21.3 ± 0	
O.aries	Neonatal	1	53.8 ± 20.1	48.4 ± 3.7
		2	47.3 ± 23.5	
		3	47.2 ± 30	
		4	45.3	
	Young	1	81.6 ± 4.7	55.5 ± 23.7
		2	49.7 ± 17.9	
		3	35.2 ± 11.6	
		4	/	

Table 3. Average of DCX+ cell cluster maximum transverse diameter in newborn *T. truncatus*, *O. aries* (A), and in young *O. aries* (B)

Table 4. Average white matter area, cluster number and density in newborn dolphin and sheep, and in young sheep.

Species	Age	Level	Area (mm^2)	Cluster number	Cluster density (clusters/mm^2)
T. truncatus	Neonatal	1	352.34 ± 39.75	3 ± 1	0.01 ± 0
		2	188.60 ± 144.11	36 ± 32	0.17 ± 0.04
		3	140.01 ± 32.59	48 ± 43	0.31 ± 0.23
		4	286.21 ± 90.62	44 ± 5	0.16 ± 0.04
O. aries	Neonatal	1	43.55 ± 9.51	30 ± 10	0.75 ± 0.45
		2	20.94 ± 2.69	7 ± 2	0.31 ± 0.05
		3	22.48 ± 1.38	2 ± 1	0.1 ± 0.06
		4	23.97 ± 2.33	0 ± 0	0 ± 0.01
	Young	1	73.94 ± 8.31	44 ± 3	0.63 ± 0.1
		2	43.52 ± 4.27	6 ± 2	0.13 ± 0.06
		3	46.72 ± 14.71	1 ± 0	0.02 ± 0.01
		4	53.76 ± 5.62	0 ± 0	0 ± 0

Table 5. Quantitave analysis of proliferating cells

Species	Age	Brain level	Proliferating cell density (Ki-67+ cells/mm^2)	Average
T. truncatus	Neonatal	1	0 ± 0	2.95 ± 2.17
		2	3.44 ± 0.62	
		3	5.21 ± 2.54	
		4	3.16 ± 1.64	
O. aries	Neonatal	1	5.32 ± 1.46	4.89 ± 1.25
		2	4.50 ± 3.06	
		3	4.32 ± 2.83	
		4	5.44 ± 4.52	

Table 6. Distance among the SVZ region and the closest DCX+ clusters in newborn *T. truncatus* and *O. aries*, and in young *O. aries*

Species	Age	Level	Distance among SVZ region and DCX+ cell clusters (µm)	Average (µm)
T.truncatus	Neonatal	1	2450 ± 0	2460 ± 220
		2	2730 ± 0	
		3	2200 ± 460	
		4	2450 ± 0	
O.aries	Neonatal	1	700 ± 0	1130 ± 690
		2	1490 ± 240	
		3	1910 ± 910	
		4	410 ± 0	
	Young	1	/	1098 ± 390
		2	1534 ± 637	
		3	770 ± 262	
		4	/	

Species	Brain development at birth		Birth/adult size BW	Myelination onset (days post-conception)		References
Dolphin (*T. truncatus*)	70%	*Advanced (40-60%)*	42,5%	Myelinated at birth	*Before birth*	Parolisi et al., 2015 Ridgway, 1990
Sheep (*O. aries*)	65%		50%	77-114 [147]		Workman et al., 2013 McIntosh et al., 1979
Mouse (*M. musculus*)	15%	*Delayed (10-30%)*	20%	25-33 [18.5]*	**Entirely after birth* ***Mostly after birth*	Workman et al., 2013 Zamenhof and van Marthens, 1976
Human (*H. sapiens*)	35%		25-29%	193-349 [270]**		Workman et al., 2013 Bonner, 1980, Dekaban and Sadowsky, 1978
Rabbit (*O. cuniculus*)	25%		10%	33-45 [31]*		Workman et al., 2013 Hahn et al., 1979

Table 7. Brain development at birth in different mammals, estimated by translation of neurodevelopmental times (see: http://www.translatingtime.net/), brain weight and myelination onset. Note that myelination onset occurs entirely after birth in mouse and rabbit, mostly after birth in humans, yet largely before birth in Cetartiodactyla (gestation period in square bracket; see also Fig. 8A). BW, brain weight.