Non-neurogenic SVZ-like niche in dolphins, mammals devoid of olfaction

Non-neurogenic SVZ-like niche in dolphins, mammals devoid of olfaction

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Abbreviated title: Absence of adult neurogenesis in dolphins

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Abstract

Adult neurogenesis has been implicated in brain plasticity and brain repair. In mammals, it is mostly restricted to specific brain regions and specific physiological functions. The function and evolutionary history of mammalian adult neurogenesis has been elusive so far. The largest neurogenic site in mammals (subventricular zone, SVZ) generates neurons destined to populate the olfactory bulb. The SVZ neurogenic activity appears to be related to the dependence of the species on olfaction since it occurs at high rates throughout life in animals strongly dependent on this function for their survival. Indeed, it dramatically decreases in humans, who do not depend so much on it. This study investigates whether the SVZ neurogenic site exists in mammals devoid of olfaction and olfactory brain structures, such as dolphins. Our results demonstate that a small SVZ-like region persists in these aquatic mammals. However, this region seems to have lost its neurogenic capabilities since neonatal stages. In addition, instead of the typical newly generated neuroblasts, some mature neurons were observed in the dolphin SVZ. Since cetaceans evolved from terrestrial ancestors, non-neurogenic SVZ may indicate extinction of adult neurogenesis in the absence of olfactory function, with the retention of an SVZ-like anatomical region either vestigial or of still unknown role.

Introduction

Adult neurogenesis is a widely-conserved feature in vertebrates, generally undergoing 'phylogenetic reduction' from amphibians to humans within tetrapods (Kempermann, 2012; Grandel and Brand, 2013). Despite remarkable discoveries leading to a better understanding of this process, the underlying logic of adult neurogenesis in evolution, as well as its function, are still a matter of debate. In all mammals studied so far, lifelong neurogenesis persists within two canonical neurogenic sites (Feliciano et al., 2015) or stem cell niches: the subventricular zone located in the forebrain (SVZ; Tong and Alvarez-Buylla, 2014) and the subgranular zone of the dentate gyrus in the hippocampus (SGZ; Vadoaria and Gage, 2014). The production of new neurons acts as a sort of 'metaplasticity' (second-level plasticity) primarily linked to learning tasks performed within specific neural systems, such as olfactory learning within the olfactory bulb (Lepousez et al., 2013; Sakamoto et al., 2014) in addition to memory and pattern separation in the hippocampus (Aimone et al., 2014; Sahay et al., 2011). However, the ultimate function/aim of adult neurogenesis as a conserved biological process is far from being identified. Substantial differences exist in the extension and importance of neurogenic sites with respect to species, age, brain region and ecological niche, thus making it difficult to identify any common traits (Barker et al., 2011; Bonfanti and Peretto, 2011; Sanai et al., 2011; Amrein, 2015; Kempermann, 2016).

In terrestrial mammals, the SVZ is the largest neurogenic site (Bordiuk et al., 2014) which provides new neurons for the olfactory bulb through the rostral migratory stream (Lois and Alvarez-Buylla, 1994). The SVZ neurogenic activity appears related to the importance of olfaction, since it occurs at high rates throughout life in animals strongly dependent on olfactory functions for their survival (e.g., rodents; Lepousez et al., 2013).Whereas in humans, who have smaller olfactory bulbs and do not depend so much on olfaction the production of new neurons dramatically decreases with age (Sanai et al., 2011). Apart from the lack of a deeper understanding of this trend, it is still unknown whether the existence of adult SVZ neurogenesis is actually dependent on olfactory functions and

related brain structures. Additionally, the search for the answer to the pivotal question of the evolutionary interpretation of the functions of neurogenesis during the tetrapod evolution still remains unanswered. Hence, in this study we investigated whether the forebrain neurogenic niche is present in natural animal models devoid of olfaction, namely, the dolphins. Marine Cetartiodactyla live underwater and have developed alternative techniques for navigation, foraging and tracking of prey (echolocation; Marriott et al., 2013). Thus, unlike terrestrial mammals and fish, they possess significantly reduced or absent olfactory systems (Breathnach, 1953; Breathnach and Goldby, 1954; Oelschläger, 2008). Even within other adult cetaceans, such as mysticetes, which possess a reduced olfactory system (Oelschläger and Oelschläger, 2009), dolphins have completely lost olfaction (Oelschläger, 2008; Cozzi et al., 2017). The terminal nerve is the only surviving component of the three functional systems, namely, the olfactory, vomeronasal, terminal systems in the nasal region of the mammalian's head (Ridgway et al., 1987). A recent report (Parolisi et al., 2015), demonstrated that neonatal dolphins lack the thick SVZ germinative layer typically persisting at birth on the ventricle wall of terrestrial mammals (Tramontin et al., 2003; Peretto et al., 2005), including humans (Del Bigio, 2011; Sanai et al., 2011). This finding might be due to either the advanced developmental stage of the dolphin brain at birth (Ridgway, 1990) or the absence of olfaction, with the possibility that periventricular neurogenesis could be absent in these aquatic mammals since birth. In addition, a recent study showed that several cetacean species have small hippocampi which do not stain for doublecortin (Patzke et al., 2015), thus indicating the possibility that adult neurogenesis itself might be lacking in these animals. Nevertheless, due to their large brain size and scarce availability of tissues that are fixed well (dolphins are legally protected animals on the basis of ethical and environmental issues), current knowledge by no means excludes the existence of postnatal neurogenesis in these animals. In the present study, the periventricular region of ten dolphins belonging to two different species (*Tursiops truncatus*, bottlenose dolphin; *Stenella coeruleoalba*, striped dolphin; Fig. 1 and Table 1) and ages (neonatal and adult) were

carefully analyzed using histology and immunocytochemistry in order to investigate the presence (or absence) of a neurogenic SVZ similar to terrestrial mammals.

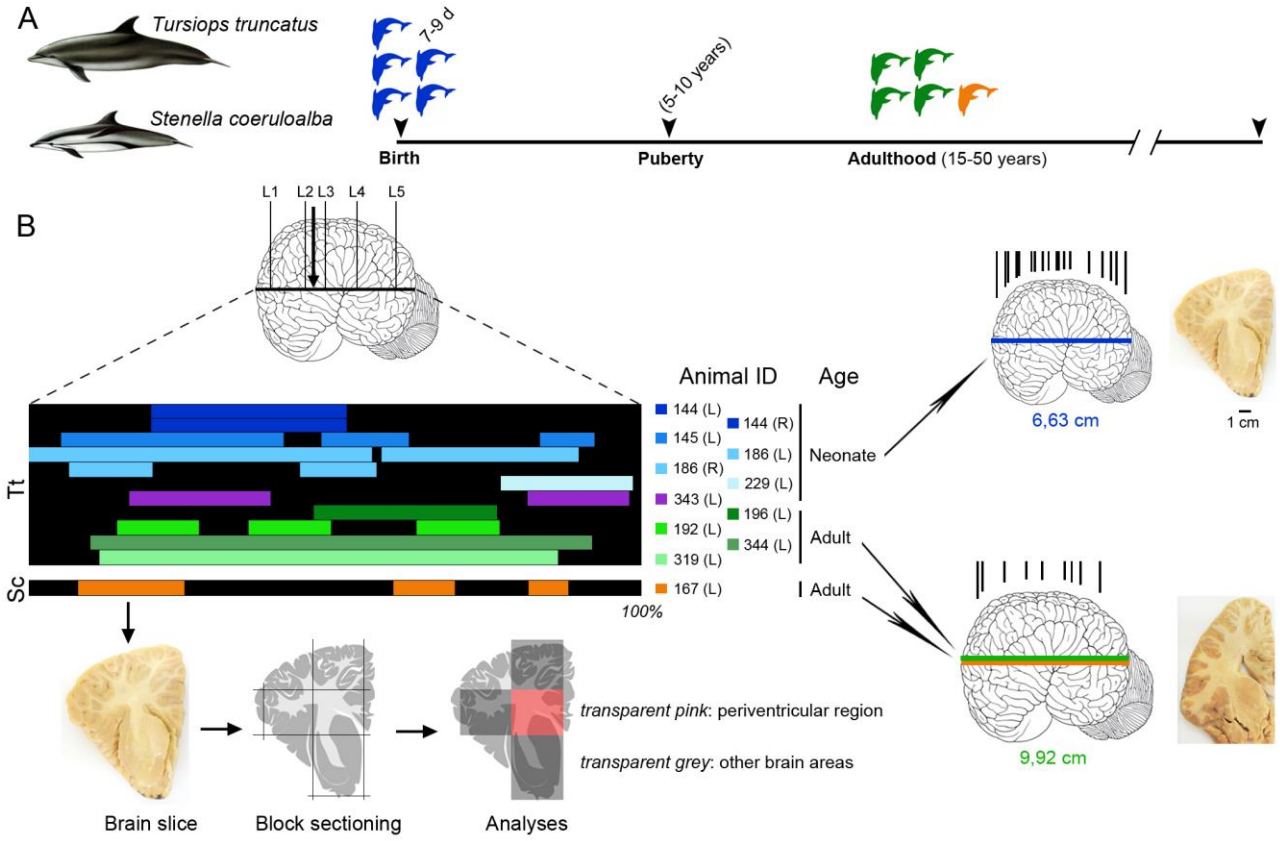

Fig 1

Materials and methods

Tissue samples

Dolphin tissues

In this study we used brain samplesobtained from 10 dolphins, 9 bottlenose dolphins (*Tursiops truncatus* Montagu, 1821 - *T. truncatus*) and 1 striped dolphin (*Stenella coeruleoalba* Meyen, 1833 - *S. coeruloalba*) stored in the Mediterranean Marine Mammal Tissue Bank (MMMTB) of the University of Padova at Legnaro, Italy (see Table 1 and Fig. 1). 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. The bottlenose and the striped dolphins have a very similar shape and anatomy. Although, differences in size and weight are evident in oceanic animals, (*T. truncatus* is generally larger than *S. coeruleoalba*) they are reduced in dolphins that live in relatively smaller basins (including the Mediterranean Sea).

Tissue samples consisted of brain coronal slices (see Parolisi et al., 2015, Morgane et al., 1980, and Fig. 1) approximately 1-1,5 cm thick, 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% buffered formalin. Post-mortem delay before actual sampling varied between a minimum of 18 to a maximum of 40 hours.

To confirm the immunodetection of Ki-67 antigen within an active germinal layer and to quantify its cell proliferation density, we sampled tissue blocks from the top of the left cerebellar hemisphere from neonatal dolphins to investigate the immunodetection of Ki-67 antigen within an active germinal layer and to quantify its cell proliferation density (see Parolisi et al., 2015).

Gross anatomy of the dolphin tissue slices

To obtain a representation of single brain levels, the anterior face of thick brain slices was photographed and imported on Neurolucida (Micro-Brightfield, Colchester,VT). Here, the outlines of each coronal section, including those of the external (pial) surface and those at the white matter/grey matter limits, were drawn (Fig. 1B). The contours were then imported to Photoshop to obtain images of each brain level. The whole procedure has been described previosly in detail (Parolisi et al., 2015).

Tissue processing for histology and immunocytochemistry

Smaller blocks were cut from thick, formalin-fixed tissue slices (about 1,5x2,5 cm; see Fig. 1 and Parolisi et al., 2015), washed in 0.1M phosphate buffer (PB), pH 7.4, for 24 hours, then cryoprotected in graded concentrations of sucrose solutions up to 30% in 0.1M PB and subsequently frozen by immersion in liquid nitrogen-chilled isopentane at -80°C. Cryostat sections (40 µm thick) were cut on glass slides treated with 3-Aminopropyltriethoxysilane (Sigma-Aldrich, 741442) and processed for histological and immunocytochemical analyses. All thick slices and relative blocks used in this study at different anterior-posterior brain levels and ages are summarized in Fig. 2.

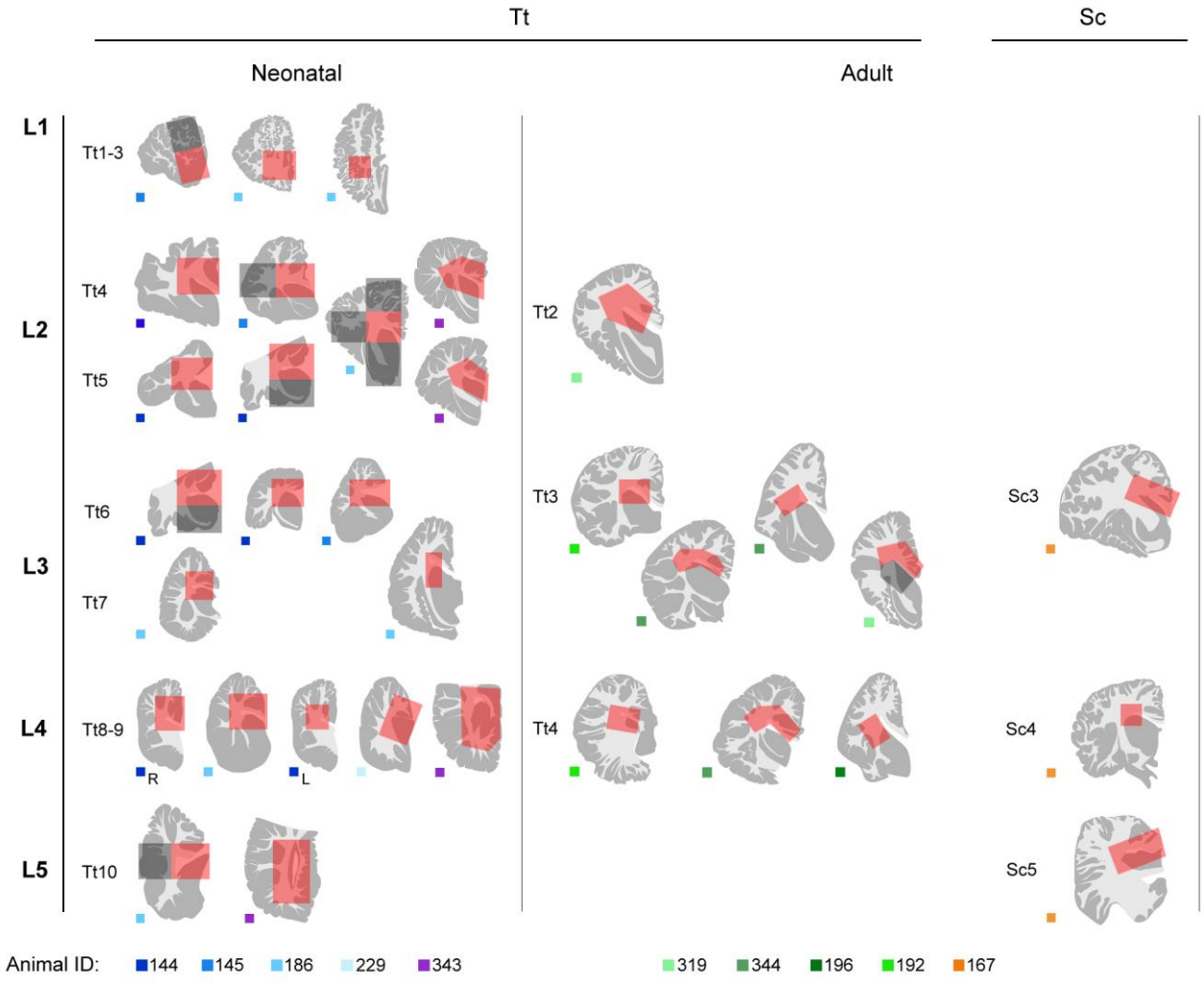

Fig. 2

For immunocytochemical analysis, two different protocols of indirect staining were employed namely, the peroxidase or the immunofluorescence techniques. In peroxidase protocol, the sections

were pre-incubated in 1% H_2O_2 - phosphate saline buffer (PBS) for 20 min, rinsed in PBS and then pre-incubated in blocking buffer (3% horse serum (HS), 2% bovine serum albumin (BSA), 1% Triton X-100 in 0.01 M PBS, pH 7.4) for 1h at room temperature to reduce non-specific staining. Then the sections were incubated for 24–48 h at 4°C in a solution of 0.01M PBS, pH 7.4, containing 0.5% Triton X-100, 2% HS, 1% BSA and the primary antibody. 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 described previously (see Ponti et al. 2006a,b), mounted with DPX Mountant (Sigma-Aldrich, 06522) and examined using an E-800 Nikon microscope (Nikon, Melville, NY) connected to a colour CCD Camera. In immunofluorescence staining the sections were rinsed in PBS and then pre-incubated in blocking buffer (3% horse serum (HS), 2% bovine serum albumin (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 24–48 h at 4°C in a solution of 0.01M PBS, pH 7.4, containing 1-0.5% Triton X-100, 2% serum, 1% BSA and the primary antibody. Following primary antisera incubation, sections were incubated with appropriate solutions of secondary cyanine 3 (Cy3)-conjugated (1:800; Jackson ImmunoResearch, West Grove, PA) and Alexa 488-conjugated (1:800; Molecular Probes, Eugene, OR) antibodies, for 2 hours RT. Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, KPL, Gaithersburg, Maryland USA), mounted with MOWIOL 4-88 (Calbiochem, Lajolla, CA). The antibodies and the dilutions used were as follows: doublecortin (DCX), polyclonal, rabbit, AbCam, 1:1000-1:1800, and polyclonal goat, Santa Cruz, 1:700; GFAP, polyclonal, rabbit, Dako, 1:2000; Ki-67 antigen, polyclonal, rabbit, Novocastra, 1:600-1:1000; vimentin (VIM), monoclonal, mouse (40EC), Exbio, 1:800; calretinin (CR), polyclonal, rabbit, Santa Cruz, 1:200, and MAP2, monoclonal, mouse, Millipore, 1:1000 (a list of antibodies tested in this study that failed to demonstrate immunostating on the dolphin tissues in the present study is provided in Table 2). To reveal the immunohistochemical and immunofluorescence reactions, the sections were

examined using an E-800 Nikon microscope (Nikon, Melville, NY) connected to a colour CCD Camera, a Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany) confocal microscope, and a Nikon Eclipse 90i. (Nikon, Melville, NY) confocal microscope.

Image processing and data analysis

All images were processed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA). Only general adjustments to color, contrast, and brightness were made. Quantitative evaluations were performed through the Neurolucida software (MicroBrightfield, Colchester, VT). The parameters considered were as follows: Ki-67+ cell density in SVZ-lr, ScWM, and corpus callosum (18 sections for neonates, 7 sections for subadult, 60 sections for adults), and in EGL (20 sections/ages); distance between lateral ventricle wall and SVZ-lr (three measures performed on 33 sections for neonates and 12 sections for adults); evaluation of the continuous gap between SVZ-lr and ScWM cell clusters (31 sections in neonates, at L3). The averages measured of the cell body diameter of SVZ-lr tightly-packed cells (20 cells) and SVZ-lr neurons (20 cells), diameter of ScWM cell clusters (181 clusters), Cm (27 measurements) and Cms areas (69 measurements) did not deviate significantly from normal distribution (Shapiro Wilk test for data $n < 30$; Anderson-Darling test for $n > 30$).

All the graphs were constructed using Graph Pad Prism (San Diego California, USA). Statistical analyses were performed by Graph Pad Prism software and included unpaired (two-tailed) Student's t test (comparing only two groups). $p < 0.05$ was considered as statistically significant. Data are expressed as averages ± standard deviation (SD).

Results

Considering the remarkable size of the adult dolphin brain (about 10 cm length and 1,3-1,7 Kg weight, in adult *T. truncatus*), and the understanding that SVZ neurogenesis is most prominent at birth in terrestrial mammals, we began the analyses on neonates, using the atlas of the neonatal/early postnatal dolphin forebrain as an anatomical reference (Parolisi et al., 2015). Careful histological screening and immunocytochemical analyses were carried out on the entire periventricular region (Fig. 2 and Table 3) in search for signs of any remnants resembling a typical neurogenic niche. Staining specificity for the cytoskeletal protein doublecortin (DCX; consistently expressed in newly generated neuroblasts and immature neurons; Nacher et al., 2001; Brown et al., 2003) and the marker for cell proliferation, namely the Ki-67 antigen (Kee et al., 2002) was confirmed by immunocytochemical detection of granule cell precursors in the external germinal layer (EGL) of the neonatal dolphin cerebellar cortex, which served as internal control (Fig. 3A). DCX staining was further tested in neonatal and adult dolphin brains by detection of a population of immature neurons occurring in the superficial layers of the cerebral cortex of most mammals studied so far (references in Bonfanti and Nacher, 2012; Fig. 3B).

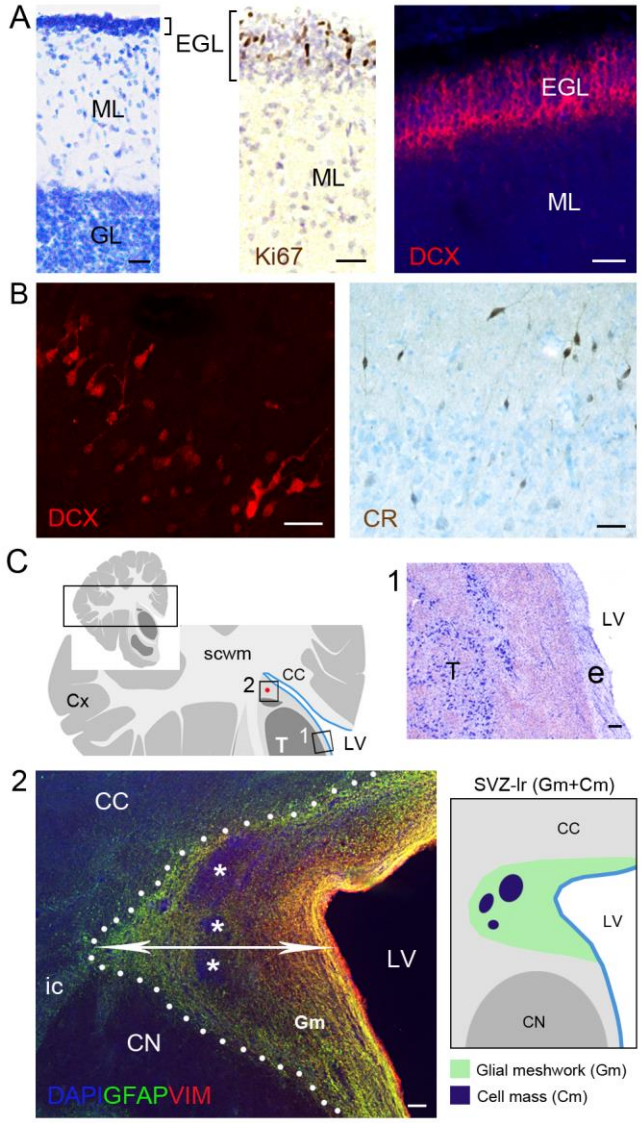

Fig 3

Identification of a SVZ-like region in neonatal and adult dolphins

Histological screening in the brain periventricular region of neonatal *T. truncatus* confirmed the absence of a well recognizable sub-ependymal germinal layer along most of the lateral ventricle wall (Parolisi et al., 2015; Fig. 3C1), yet clusters of small tightly-packed cells (3,4-0,63 µm - cell body diameter) were detected in a restricted region located at its dorsolateral corner, from level Tt3 to Tt10 (Figs. 3C2 and 4). Systematic analysis carried out on serial sections (see Table 3 for anteroposterior brain level steps) revealed that these clusters form a very thin, continuous cell mass

(Cm; 145615,06±68402,16 μm^2 - average area at L2-L4 levels; see Table 4) lining the entire lateral ventricle extension and reaching a length of approximately 4,9 cm (estimated by considering consecutive brain sections cut following the beak-fluke axis that contained the Cm; see Morgane et al., 1980 and Fig. 4). Immunocytochemical detection of the astrocyte marker glial fibrillary acidic protein (GFAP) revealed a dense glial meshwork (Gm) completely surrounding the Cm, sharply ending at the limit with the corpus callosum (dorsal and lateral) and the forebrain caudate nucleus (lateral and ventral; Figs. 3 and 4). The Cm was never observed to be directly in contact with the ventricular wall, maintaining an evident distance from the ependyma (Fig. 4 and below). At the most anteroposterior brain levels, the Cm was split in smaller cell clusters (Cms; 6210,76±3866,14 μm^2 - average area at L1 and L5 levels), their number varying from 1 to 12, being higher at the extremities and lower in the middle (Fig. 4 and Table 4). The neuroblast-like nature of these cells was suggested by their DCX expression (Fig. 4A). On the whole, this region appears to be organized into two main cellular compartments (Cm and Gm) that seem phylogenetically related to the adult SVZ described in most terrestrial mammals (Lois et al., 1996; Peretto et al., 1997,2005; Bonfanti and Peretto, 2011), and thus referred hereafter to as SVZ-like region (SVZ-lr).

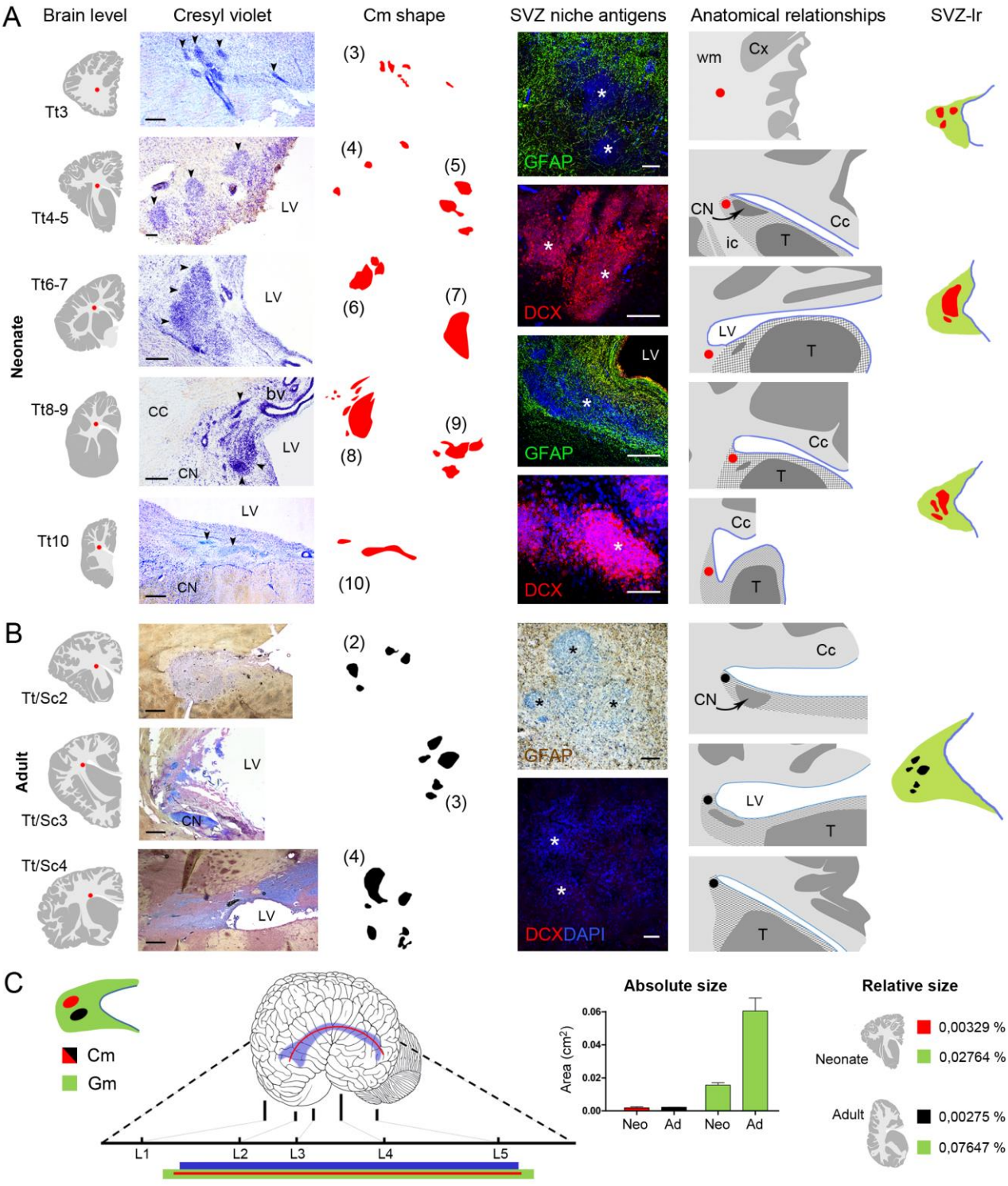

Fig 4

An SVZ-lr similar to that of neonates, sharing the same location, was identified in adult dolphins belonging to both species (Fig. 4B). Analysis of the SVZ-lr total area and Cms area in neonatal and adult brains revealed a slight increase in size for the SVZ-lr in adults with respect to neonates, whereas no major changes were observed in the Cm size through ages (Fig. 4C and Table 4).

Periventricular neurogenic processes in dolphins are almost exhausted at birth and absent in adulthood

Immunocytochemical detection of Ki-67 antigen revealed only a few scattered proliferating cells in the whole SVZ-lr of neonatal animals (Fig. 5A) and none in adults. In the neonatal SVZ-lr the Ki-67+ nuclei appeared evenly distributed both in Cm and Gm, their frequent appearance in doublets indicative of the absence of cell migration. Quantitative analysis revealed very low rates of cell proliferation (average cell density/mm^2 43,16±32,92; Table 4) substantially similar to those in the surrounding parenchymal tissue (Fig. 5B). In the young *T. truncatus* (subadult), such rate was even lower than in the corpus callosum (Fig. 5B and Table 4) wherein a low, protracted proliferation of glial cell precursors is known to occur (Dawson et al., 2003). The number of proliferating cells in the neonatal dolphin SVZ-lr was found to be negligible when compared with those typically found in the correspondent neurogenic site of terrestrial mammals (average cell density/mm^2 2657±86, see Armentano et al., 2011 and Fig. 5B, right). This is possibly indicative of the precocious exhaustion of neurogenic activity at birth.

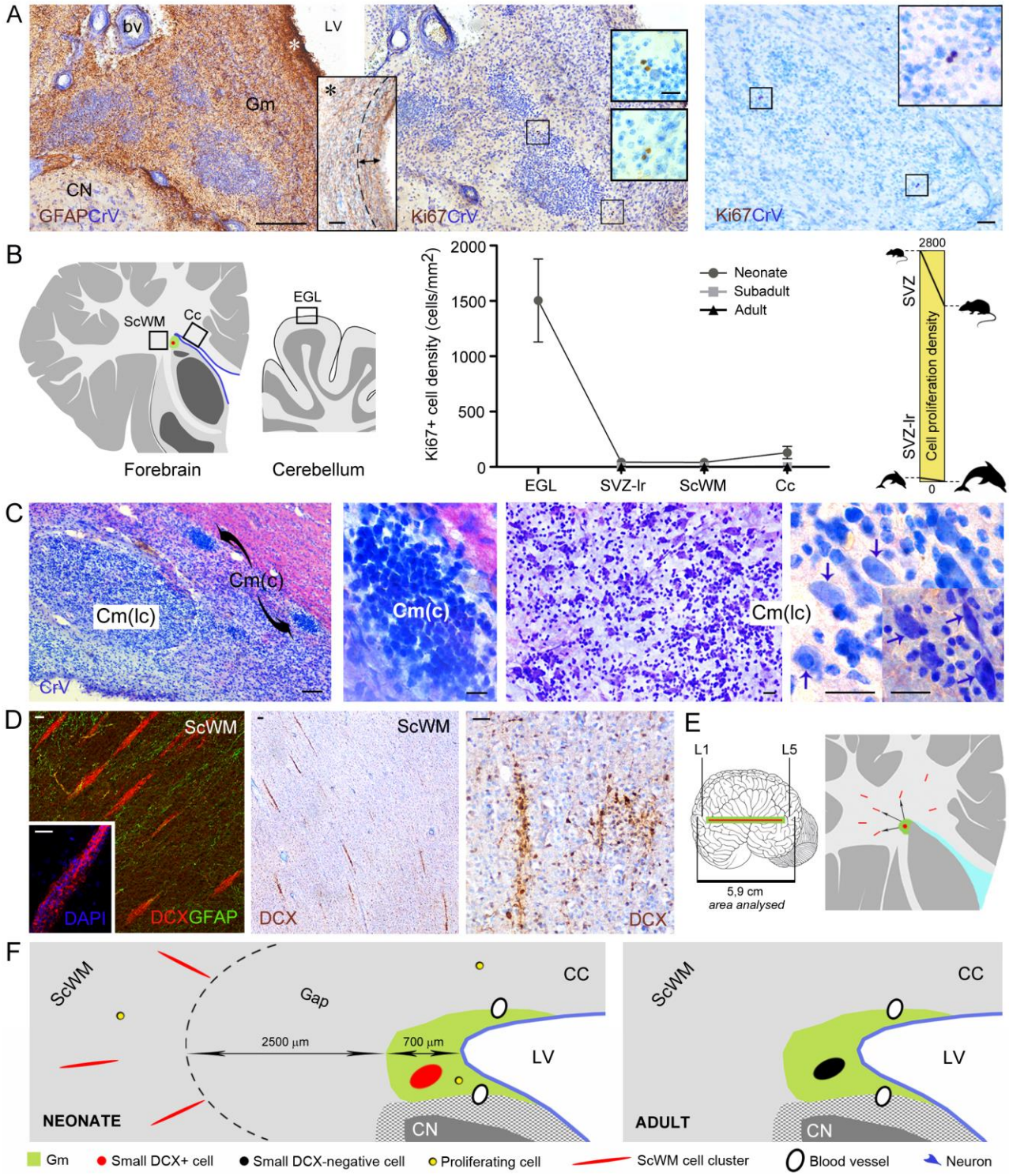

Fig 5

Other features previously unnoticed in terrestrial mammals were also observed in the internal organization of the Cms. Some of the cell clusters appeared "less compact", with small-sized cells more sparse and distant to each other (Fig. 5C). In the neonatal SVZ-lr, many of these cells were not expressing DCX (Fig. 6A) and were intermingled with larger cells morphologically

recognizable as neurons with triangular- or bipolar-shaped soma (5,74±1,36 µm - cell body diameter; Fig. 5C). Most of these cells were immunoreactive for the neuronal marker microtubule-associated protein 2 (MAP2; Herzog and Weber, 1978; Fig. 6), whereas, a smaller population (around 15-30% - value estimated on 5 sections for each age performed at the L3-L4 levels) expressed the calcium-binding protein calretinin (CR; von Bohlen and Halbach, 2011; Fig. 6; see Fig. 3B for internal control). The partial lack of DCX staining, along with the expression of neuronal maturation markers, strongly confirm a progressive loss of neurogenic activity in the dolphin SVZ-lr starting at very early ages. Similarly, no DCX staining was detectable in the SVZ-lr of adult dolphins (Fig. 4B and 6A), wherein the CR+ neurons showed further signs of differentiation, i.e. the extension of neuritic processes (Fig. 6).

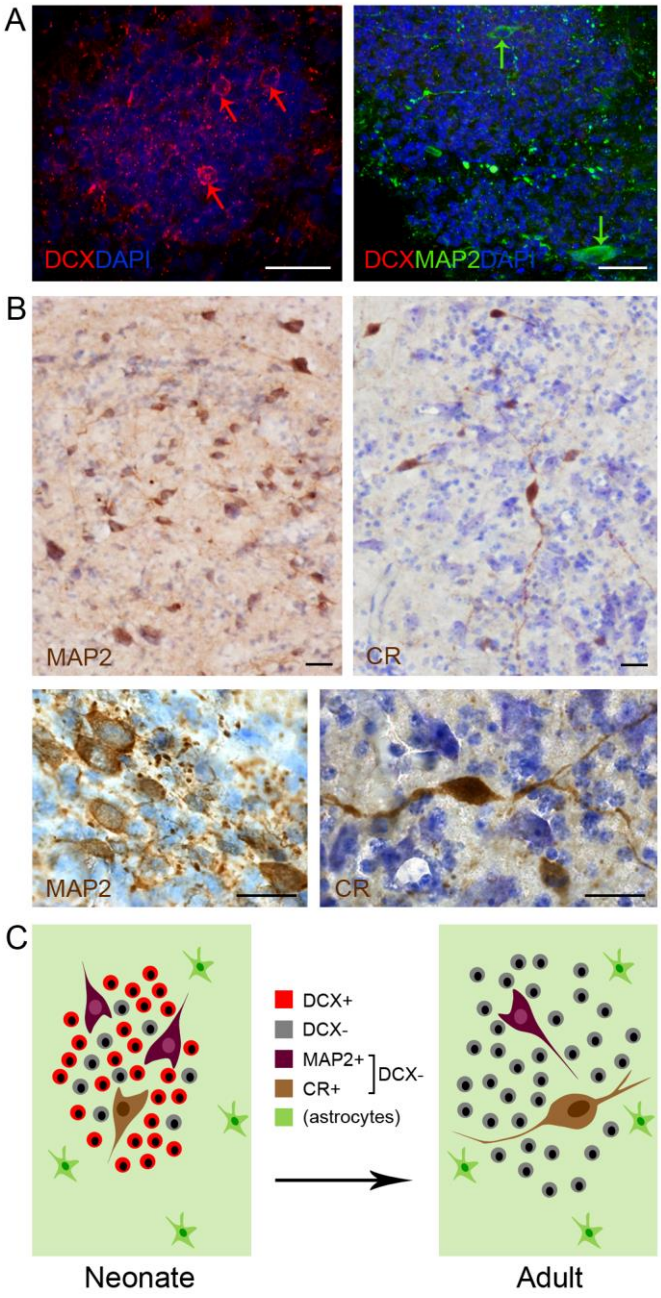

Fig 6

SVZ neurogenesis provides neuronal precursors for the olfactory bulb in all terrestrial mammals (Lois and Alvarez-Buylla 1994; Bonfanti and Ponti, 2008). Thus, the occurrence of an SVZ-lr in the brains of aquatic mammals raises the question as to whether some streams do exist in spite of the absence of olfaction/olfactory bulb in these animals. To answer this question, the subcortical white matter (ScWM) surrounding the entire SVZ-lr was analyzed at all ages in search for DCX+ cells/streams. In the neonates, elongated clusters of small, tightly-packed cells were detectable (Fig. 5D). Both compact, thick and less compact, thin clusters (37,52±35,47 µm - transverse diameter,

with substantial variability in different animals) were observed (Fig. 5D). They were mostly radially-oriented in large portions of the ScWM, occupying a fan-shaped area along the inner part of the emisphere (anteriorly, laterally, ventrally, and posteriorly to the SVZ-lr), yet never reaching the cortex. Since the shape of these structures might be somehow reminiscent of the "parenchymal chains" of neuroblasts previously described in other mammals (Luzzati et al., 2003; Ponti et al., 2006a), they were investigated for possible presence of dividing cells and/or connection with the SVZ-lr and its Cms. No Ki-67+ cells were ever detectable in association with the ScWM cell clusters, although a few proliferating cells were occasionally found in the tissue among the clusters (Fig. 5F). Upon careful analysis carried out all along the SVZ-lr (see Fig. 5E and Table 3 for serial section steps), it was found that no direct connections ever occurred between the SVZ-lr and any of the ScWM cell clusters. Rather, a continuous "gap" completely devoid of cell clusters (2500±200 µm thick; Fig. 5E,F) was present in the areas surrounding the SVZ-lr, in every direction, including anterior and posterior aspects, thus excluding the possibility that they are continuous streams of cells generated within the SVZ-lr.

Discussion

The brains of all terrestrial mammals host a remnant of the periventricular, embryonic germinal layer (SVZ) particularly prominent at birth (Tramontin et al., 2003; Peretto et al., 2005) and persisting throughout life as a major neurogenic site (Kriegstein and Alvarez-Buylla, 2009; Bordiuk et al., 2014). Here we show that dolphins, although lacking such a layer, host a very small SVZ-lr located at a remote tip of the lateral ventricle, which can be consistently found at neonatal and adult ages. The SVZ-lr occupies an area approximately similar the real size of its counterpart in mice

(Fig. 4C), whose brain, in comparision, is 40 fold smaller if a correspondent coronal section area is measured, and 3000 fold smaller if the weight or volume are considered (Rose et al., 2006; Marino et al., 2000).

Unlike the SVZ of terrestrial mammals (Tramontin et al., 2003; Lois et al., 1996; Peretto et al., 1997,2005), the SVZ-lr of dolphins is already compartmentalized soon after birth with its structure being reminiscent of adult neurogenic sites (Ponti et al., 2006a; Bonfanti and Ponti, 2008). This phenomenon, although unusual in mammals, fits well with the highly advanced developmental stage of the brain in neonatal aquatic mammals (Parolisi et al., 2015), which is related to the immediate need of the newborn to already possess all the swimming competences required for life, including the ability to reach the surface and breathe (Ridgway, 1990). Yet, it is surprising that a brain region sharing features (location, inner histological organization and some molecular aspects) with the SVZ neurogenic niche of terrestrial mammals does persist in dolphins, apparently in contrast with the absence of olfaction/olfactory structures. What appears to be unique in this SVZ-lr is its extremely low rate of cell proliferation detectable in neonates, followed by utter disappearance. The density of dividing cells revealed by Ki-67 antigen localization in the SVZ-lr of the neonatal dolphins (43,16±32,92) is 34 fold lower when compared with the germinal layer of the cerebellar cortex in the same animals (1504,63±374; Figs. 3 and 5 and Table 4), 62 fold lower than that existing in the SVZ of neonatal rodents (2657±86; Armentano et al., 2011), 47 fold lower than in adult rodents (2018,5±420; Rolando et al., 2012; Fig. 5), and it is not higher than in the surrounding brain parenchyma (Fig. 5B). Even in humans, despite a dramatic reduction of SVZ thickness with age (Sanai et al., 2011), a highly proliferative region exists in neonates, which persists to a lesser extent in adult and old individuals (Eriksson et al., 1998; Sanai et al., 2004; Wang et al., 2011).

The very early exhaustion of peri-ventricular neurogenic activity in dolphins is also reflected by the cellular and molecular features of the Cms in the SVZ-lr. In neonates, the small, neuroblast-like

cells are not tightly-packed, show variable and incomplete DCX staining and are intermingled with neurons expressing mature neuronal markers such as MAP2 and CR (Figs. 5-7).

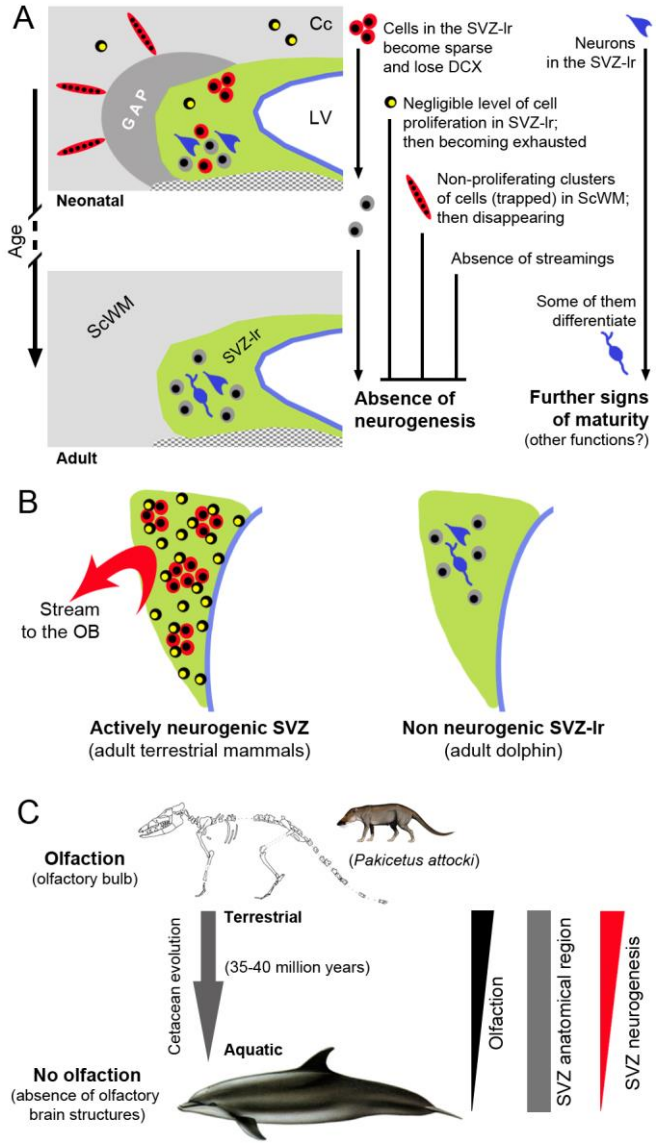

Fig 7

In adults, no DCX staining is detectable, whereas the SVZ-lr neurons are still detectable, a subpopulation of them showing further traits of differentiation such as the extension of neuritic processes (Fig. 6). Hence, in the dolphin SVZ-lr an early exhaustion of cell division goes in parallel with neuronal maturation. Such differentiation "in situ" might simply be a consequence of the cellular/molecular environment of the SVZ-lr (e.g., absence of any continuous supply of new, young neuroblasts) which is no more supportive as an active stem cell niche. These observations are in sharp contrast with the current knowledge on the SVZ of all terrestrial mammals, characterized

by an embryonic-like tissue which persists into adulthood (Fig. 7), although with different degrees of proliferative activity from rodents to humans (Ponti et al., 2013; Eriksson et al., 1998; Wang et al., 2011; Sanai et al., 2004,2011). Additionally, a careful analysis extended to the brain regions surrounding the SVZ-lr did not reveal any streams of cells spanning from the periventricular Cms to any other direction, unlike terrestrial mammals which all exhibit a marked rostral migratory stream at perinatal stages (Lois and Alvarez-Buylla 1994; Peretto et al., 2005). Although the radially-oriented, DCX+ cell clusters present in the ScWM of neonates were reminiscent of "chain-like" structures (Luzzati et al., 2003; Ponti et al., 2006a), the occurrence of a continuous white matter gap (absence of any direct contact with the SVZ-lr perimeter) along with the scarcity of cell divisions in the SVZ-lr itself, exclude the possibility that they can represent any product of an ongoing neurogenic activity.

Once it is established that in dolphins all SVZ neurogenic processes are substantially exhausted at birth, clusters of DCX+ cells still present in the SVZ-lr and white matter of neonatal animals are a matter of further investigation. The occurrence of DCX+ cells in the periventricular white matter or in the corpus callosum has been previously shown in, large-brained mammals at postnatal ages (Fung et al., 2011). Although DCX is commonly expressed in newly generated neuroblasts (Brown et al., 2003), staining for this cytoskeletal protein alone is not at all a proof for the occurrence of neurogenesis, since DCX is heavily present in non-newly generated adult cell populations (Gomez-Climent et al., 2008; Luzzati et al., 2009; Bonfanti and Nacher, 2012). Considering the extremely rapid developmental growth of the dolphin brain and its remarkably advanced stage of maturation at birth (Ridgway, 1990; Parolisi et al., 2015) the ScWM cell clusters appear to be previously migrating streams of cells "trapped" in the thick white matter which fills the central part of the hemispheres, as sort of "remnants" of the last neurogenic wave. In fact, no more DCX+ cells are detectable in the entire ScWM of adults.

In this study, morphological, antigenic, proliferative aspects converge to support the conclusion that the dolphin SVZ-lr is a vestigial structure not behaving as an active neurogenic site since very early

postnatal stages (Fig. 7). This finding is consistent with previous studies that demonstrate that several cetacean species have small hippocampi which do not stain for DCX (Patzke et al., 2015), and strongly indicate that adult neurogenesis is totally lacking in dolphins. The two main findings of this study that can have evolutionary considerations are: 1) the lack of clear signs of active neurogenesis in aquatic mammals devoid of working olfaction/olfactory brain structures, and 2) the counterintuitive existence of an SVZ-like region throughout their lifespan. The former observation supports the occurrence of a strict relationship between adult SVZ neurogenesis and olfaction, confirming the hypothesis that in mammals the production of highly specific populations of new neurons is selectively destined for physiological roles such as learning, memory and plasticity (Bonfanti, 2011; Peretto and Bonfanti, 2014; Obernier et al., 2014). On the other hand, the persistence of an anatomical region reminiscent of the SVZ neurogenic niche (in fact, non-neurogenic at all) plays against the simple hypothesis that a mammal lacking olfaction should not possess an SVZ-like region. The explanation for this might be found in the evolutionary history of these animals. Dolphins, and more in general cetaceans, evolved from terrestrial artiodactyls that returned to the sea 35-40 million years ago (Thewissen et al., 2001). Data from fossil studies show that the terrestrial ancestors of dolphins were wolf-sized terrestrial carnivorous (*Pakicetus*) endowed with olfactory structures (Gingerich et al., 1983; Kishida et al., 2015). Then, in the early Eocene period, by undergoing a gradual and branched transition from land to sea, they lost the capacity to perceive odors (Gingerich et al., 1983; Thewissen et al., 2001). Although dolphin foetuses possess small olfactory structures, they regress completely shortly after birth (Buhl and Oelschläger, 1988; Cozzi et al., 2017). Adult dolphins only possess the terminal nerve, originating from the olfactory placode and reaching the basal telencephalon (Buhl and Oelschläger, 1988). While in adult terrestrial mammals, including man, the terminal system is reduced to a few hundred neurons, in adult bottlenose dolphins (and other delphinid species), fiber strands and interspersed ganglia enter the olfactory tubercle and the pre-piriform cortex (Ridgway et al., 1987). Yet, apart from its common developmental origin with the olfactory system, the terminal nerve system is

completely independent from the SVZ germinal layer, both anatomically and functionally (Buhl and Oelschläger, 1986).

Therefore, the retention of the SVZ-lr in extant dolphins as an anatomical region having lost any neurogenic capacity strongly suggests a slow extinction of adult neurogenesis in mammals not dependent on olfaction for survival. The findings of the present study also open up the possibility that non-neurogenic SVZ could have changed its role over time, from neurogenesis to new, yet unknown roles. However, the latter aspect would hardly be an object of investigation in ethically protected animals such as the toothed whales, unless new methods of analysis are developed in the future.

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631

632 **Figure legends**

633

634 **Figure 1.** Animals and brain tissue samples used in this study (see also Table 1 and Parolisi et al.,
635 2015). A, Ten specimens belonging to two species of dolphins (*T. truncatus*, Tt; *S. coeruleoalba*,
636 Sc) at different ages (same colours as in B) were used. B, Arrow, coronal cutting direction to obtain
637 thick brain slices (examples on the right). ID, identification numbers; L, left hemisphere; R, right
638 hemisphere. Coloured lines indicate the amount of tissue available for
639 histological/immunohistochemical analyses in each animal and hemisphere (neonatal Tt, shades of
640 blue; adult Tt, shades of green; adult Sc, yellow), as a percentage of the whole brain extension
641 (black backclot; not in scale).

642

643 **Figure 2.** Tissue blocks analysed at different brain levels in all animals and ages (colors explained
644 in Fig. 1B).

645

646 **Figure 3.** Identification of an SVZ-like region (SVZ-lr) in the neonatal dolphin brain (*T. truncatus*)
647 and internal controls based on cell populations typically identified by DCX, CR, and Ki-67 antigen
648 in cerebral and cerebellar cortices of the same animals. A, Actively proliferating granule cell
649 precursors in the external germinal layer (EGL) of neonatal, as an internal control for Ki-67 antigen;
650 GL, granule cell layer; ML, molecular layer. B, Cortical neurons as an internal control for DCX (see
651 Bonfanti and Nacher, 2012) and calretinin (CR). C, No signs of residual germinal layer are
652 detectable along the lateral ventricle wall (1). General features reminiscent of the terrestrial
653 mammal SVZ are recognizable in a very small region (2), comprised between caudate nucleus
654 (CN), corpus callosum (CC) and ventricular corner: cell masses composed of tightly-packed cells
655 (Cm, asterisks) are surrounded by a dense, GFAP+, Vim+ astrocytic glial meshwork (Gm); Gm and

Cm form the area referred to as SVZ-lr (dotted line on the left; green area on the right). T, thalamus; Cx, cortex; ic, internal capsule; scwm, subcortical white matter. Scale bars: 50 µm.

Figure 4. Histological and immunocytochemical characterization of the SVZ-lr in neonatal and adult dolphins (*T. truncatus* and *S. coeruloalba*). A, Topographical position of the Cms (small red dots, left), their profile (red areas, middle), and their detailed neuroanatomical location (right) at different anterior-posterior brain levels. Cms are indicated by arrowheads in CrV stained sections and by asterisks in immunofluorescence images; most of the small, tightly-packed cells are DCX+ and are surrounded by a GFAP+ astrocytic glial meshwork (Gm, green in the schematic drawings on the right, illustrating the compartmentalized architecture of the SVZ-lr); CN, caudate nucleus; T, thalamus; LV, lateral ventricle; bv, blood vessels. B, Same analyses carried out on brains of adult animals, in both species; the profile of the Cms is black, since no DCX staining is detectable in their cells. C, Left, anterior-posterior extension of the SVZ-lr in the neonatal dolphin (not showed in adults since very similar); in blue, the lateral ventricle. Right, absolute and relative size of the dolphin SVZ-lr and its Cms (absolute size: areas measured on 33 sections for neonates and 12 sections for adults; relative size: % absolute area with respect to coronal brain slice area; analysed at L2) at different ages; the SVZ-lr is slightly enlarged in adults whereas the Cms are substantially unchanged. Scale bars: A, 200 µm (right bottom, 50 µm); B, left, 1000 µm; right, 50 µm.

Figure 5. Estimation of neurogenic activity in the SVZ-lr and surrounding parenchyma of neonatal (A, C left, D) and adult (C, right) dolphins. A, Left, no particular density of astrocytic cells forming the glial meshwork (Am) is detectable close to the ventricular wall (the darker area is an optical effect due to thickness of the brain section; see inset); middle and right, a few scattered dividing cells revealed by Ki-67+ nuclei are detectable in the whole SVZ-lr area of neonatal dolphins, randomly distributed in the Cms, at their limits or in the Gm. B, Quantification of proliferating cell density in the dolphin SVZ-lr, subcortical white matter (ScWM), and corpus callosum (Cc) at

different ages; squares indicate the areas analysed; cell division rate is very low in the SVZ-lr of neonates, substantially matching that in the parenchymal tissue (middle), being 34 fold lower than in the cerebellar external germinal layer (EGL) and 62 fold lower than in the SVZ of neonatal mice (right). No cell division is detectable in adults (graphically represented in F). C, At all ages and species, in the SVZ-lr both compact (c) and less compact (lc) cell masses (Cm) are present, the latter also harboring large cells with neuronal morphology (right, blue arrows). CN, caudate nucleus; CrV, cresil violet; bv, blood vessels. D, Clusters of tightly-packed, DCX+ cells in the ScWM surrounding the SVZ-lr in neonatal dolphins; their size and compactness is variable in different individuals. After serial analysis at different brain levels (E, right; see Table 3), clusters were confined within the ScWM and no one can be ever found in direct contact with the SVZ-lr (or its Cms), a gap always existing between the most inner clusters and the SVZ-lr perimeter (F, left). No DCX+ cell clusters are detectable in the white matter of adults (F, right). Scale bars: A left, middle, 100 µm, right, 30 µm, insets, 10 µm; C left, 50 µm, others, 10 µm; D, 20 µm.

Figure 6. Cellular organization of the Cms in the dolphin SVZ-lr at different ages (A, left and B, left bottom: neonate; A, right, B, top and right bottom: adult). A, Only scattered, small round-shaped cells are DCX+ in a Cm of a neonatal dolphin (left), and none in the adult (right). B, Most cells with large cell bodies found in the SVZ-lr are MAP2+ neurons (B, left, top and bottom; see also A, right); a smaller amount of neurons is CR+, showing more differentiated shapes in adults, including neurite extensions (B, right, top and bottom). C, Schematic representation of the cell types in the dolphin SVZ-lr at different ages: small, round-shaped cells in the Cms show an overall loss of DCX staining shifting from young to adult ages; neurons are already present around birth, although showing less mature morphologies than in the adult (*T. truncatus*). Scale bars: 20 µm.

Figure 7. SVZ-lr and neurogenesis in dolphins at different ages: comparison with terrestrial mammals. A, Several features displayed by the SVZ-lr of neonatal and adult dolphins converge to

the conclusion that their periventricular neurogenesis is almost exhausted at birth then being absent, with progressive neuronal differentiation occurring within the SVZ-lr itself. Green, glial meshwork; red, DCX+ cells; grey, DCX-negative cells; yellow dots, cell proliferation; ScWM, subcortical white matter. B, Striking contrast between the typical proliferative, neurogenic SVZ of terrestrial mammals and the non-neurogenic SVZ-lr of dolphins. C, Evolutionary considerations and hypotheses: dolphins are cetaceans devoid of olfaction which derive from terrestrial mammals endowed with olfactory structures (wolf-sized *Pakicetus*, Thewissen et al., 2001; Kishida et al., 2015); an SVZ-lr (intended as an anatomical region) has been retained in extant dolphins, yet losing any neurogenic capacity.

720 **Table 1.** Detail of the sampled bottlenose dolphins. C.E., Controlled environment.
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Specimen	ID	Sex	Origin	Length/Weight	Age
T. truncatus	186	F	C.E.	110,5 cm/19kg	(neonatal) 19 days
	145	M	C.E.	118 cm/19kg	(neonatal) 9 days
	144	M	C.E.	117 cm/22,1kg	(neonatal) 9 days
	229	M	C.E.	99 cm/19 kg	(neonatal) 7 days
	343	F	C.E.	95 cm	(neonatal) 1 day
	344	M	Stranded	195 cm/98,5 Kg	Subadult
	192	F	Stranded	240 cm/178,5 kg	Adult
	196	M	Stranded	300 cm/219 kg	Adult
	319	M	Stranded	310 cm	Adult
S. coeruleoalba	167	M	Stranded	198 cm/94 kg	Adult

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Table 2. Antibodies tested in this study, not working on our dolphin tissues.

Antigen	Antibody/antiserum	Host	Diluition	Source
BLBP	poly	rabbit	1:1000	Chemicon
Calbindin	poly	rabbit	1:10000	Swant
Calbindin	mono	mouse	1:1000	Swant
CdIIb	mono	mouse	1:1000	Sigma
DCX	poly	guineapig	1:800	Millipore
GABA	poly	rabbit	1:2000	Sigma
GFAP	mono	mouse	1:100	Millipore
GST	poly	rabbit	1:500	MBL
Iba 1	poly	rabbit	1:1000	Wako
IIIBtub	poly(Tuj1)	rabbit	1:1000	Covance
IIIBtub	mono (Tuj1)	mouse	1:100	Millipore
Ki-67	mono	mouse	1:300	BD Pharmigen
Ki-67	mono	mouse	1:200	Dako
Laminin	poly	rabbit	1:800	Dako
Map5	poly	goat	1:600	Santa Cruz
Map5	Mono	mouse	1:1500	Chemicon
MBP	mono	mouse	1:100	Millipore
NeuN	mono, A60	mouse	1:200	Millipore
Neurofilament	poly	rabbit	1:800	AbCam
Ng2	mono	mouse	1:200	Chemicon
Ng2	mono	mouse	1:300	US Biological
Ng2	mono	mouse	1:200	Upstate
Ng2	mono	mouse	1:200	Sigma
Ng2	poly	rabbit	1:400	Chemicon
Olig2	poly	goat	1:400	R&D System
Olig2	poly	rabbit	1:500	Millipore
PVim	poly	ra	1:800	AbCam
Parv19	mono	mouse	1:2000	Swant
Parv19	poly	rabbit	1:3000	Sigma
Pax 2	mono	mouse	1:800	Santa Cruz
Pax6	poly	rabbit	1:800	AbCam
PDGFRa	poly	rabbit	1:1000	Santa Cruz
PDGFRa	poly	rat	1:100	BD Pharmigen
PH3	poly	rabbit	1:500	Millipore
PH3	mono	mouse	1:300	Millipore
PSA NCAM	mono	mouse	1:900/1:2000	Biocampare
RIP	mono	mouse	1:400	Chemicon
S100B	poly	rabbit	1:3000	Swant
S100B	mono	mouse	1:10000	Sigma
Sox10	poly	goat	1:800	AbCam
Sox2	poly	rabbit	1:1000	Millipore
Sox2	poly	goat	1:400	Santa Cruz
Sox9	poly	rabbit	1:1000	Millipore
Tbr1	mono	mouse	1:600	AbCam
Tbr2	poly	rabbit	1:800	Millipore
Tenascin C	poly	rabbit	1:500	AbCam

Table 3. Step intervals (µm) between cryostat tissue sections used for different types of analysis within and outside the SVZ-lr of the dolphin brains (see Fig. 2).

Species *Age*	**Brain level**	**SVZ-like region**				**ScWM and Cc**	
		Cm (CrV)	**Gm** (GFAP)	**Cm** (DCX)	**Cell proliferation** (Ki-67)	**ScWM clusters** (CrV, DCX)	**Cell proliferation** (Ki-67)
Tt *Neonate*	2	400	400	400	800	600	800
	3	400	200	200	400	400	400
	4	300	200	200	400	200	400
	5	300	400	400	400	200	400
	6	320	400	400	420	200	420
	7	320	400	400	420	400	420
	8	320	400	400	420	400	420
	9	400	400	400	800	600	600
	10	400	400	400	800	600	800
Tt *Adult*	*2*	800	1800	1200	2000	1200	1200
	3	400	1800	800	2000	800	800
	4	400	1800	800	2000	1000	1000
Sc *Adult*	*3*	400	1800	800	2000	1200	1200
	4	400	1800	800	2000	800	800
	5	400	1800	1200	2000	1000	1000
Analysed sections		**879**	**633**	**738**	**459**	**1194**	**811**

Tt, *T. truncatus*; Sc, *S. coeruloalba*; Cm, periventricular cell mass; Gm, glial meshwork; ScWM, subcortical white matter; Cc, corpus callosum; CrV, cresil violet; DCX, doublecortin; GFAP, glial fibrillary acidic protein.

Table 4. Cellular and molecular features of the SVZ-lr in neonatal (1 day and 7-9 days) and adult *T. truncatus*.

Species *age*	**Brain level**	**Cell masses (Cm)**				**Astrocytic meshwork (Am)**		**Ki-67+ cells** (cells/mm²)
		Number	**Total area** (μm²)	**DCX**	**N**	**GFAP**	**Area** (μm²)	
Tt *Neonatal (1 day)*	**L2**	11-10	139204,69±50298,49	+	+	+	2915883,33 ±128405,96	28,17±10,30
	L3	12-4	203093,03±38014,69	+	+	+	2235382 ±270675,11	n.d.
	L5	7-3	120030,02±51137,13	+	+	+	1141864,60 ±435986,77	20,07±10,05
Tt *Neonatal (7-9 days)*	**L1**	6-4	53028,95±20979,75	+	-	+	n.d.	n.d.
	L2	5-3	64972,45±25643,77	+	-	+	452090,11±87860,12	n.d.
	L3	3-1	158851,38±84676,47	+	+	+	876197,75±24983,08	78,57±84,52
	L4	2-6	91068,19±13325,03	+	+	+	688922,50±142514,73	31,35±5,20
	L5	2-6	118389,6 ±27084,12	+	-	+	4705096 ±1893947	56,55±22,45
Tt *Adult*	**L2**	6-8	222255,90 ±2218,19	-	+	+	7145366,66 ±783523,72	No cells
	L3	4-8	217682,52 ±6467,73	-	+	+	6044218 ±778629,68	
	L4	7-5	65976,75 ±29580	-	+	+	4621066,66 ±1570835,62	