The Cerebral Cortex of the Pygmy Hippopotamus, *Hexaprotodon liberiensis* (Cetartiodactyla, Hippopotamidae): MRI, Cytoarchitecture, and Neuronal Morphology

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ABSTRACT

The structure of the hippopotamus brain is virtually unknown because few studies have examined more than its external morphology. In view of their semiaquatic lifestyle and phylogenetic relatedness to cetaceans, the brain of hippopotamuses represents a unique opportunity for better understanding the selective pressures that have shaped the organization of the brain during the evolutionary process of adaptation to an aquatic environment. Here we examined the histology of the cerebral cortex of the pygmy hippopotamus (*Hexaprotodon liberiensis*) by means of Nissl, Golgi, and calretinin (CR) immunostaining, and provide a magnetic resonance imaging (MRI) structural and volumetric dataset of the anatomy of its brain. We calculated the corpus callosum area/brain mass ratio (CCA/BM), the gyrencephalic index (GI), the cerebellar quotient (CQ), and the cerebellar index (CI). Results indicate that the cortex of *H. liberiensis* shares one feature exclusively with cetaceans (the lack of layer IV across the entire cerebral cortex), other features exclusively with artiodactyls (e.g., the morphology of CR-immunoreactive multipolar neurons in deep cortical layers, gyrencephalic index values, hippocampus and cerebellum volumetrics), and others with at least some species of cetartiodactyls (e.g., the presence of a thick layer I, the pattern of distribution of CR-immunoreactive neurons, the presence of von Economo neurons, clustering of layer II in the occipital cor-
INTRODUCTION

Although all vertebrate brains appear similar at certain stages of embryonic development (Puelles and Rubenstein, 2003), developmental differences occur beyond the embryonic period and their functional significance is still poorly understood. Vertebrates display a tremendous variation in brain structure including brain size, degree of folding of the cerebral cortex, size of cortical fields (the number of which, however, remains rather comparable among species), and cortical organization (Striedter, 2005). Thus, in the context of brain evolutionary studies, it becomes pivotal to understand how adaptation to different environments modifies brain structure. The clade Cetartiodactyla includes both terrestrial (artiodactyls) and fully aquatic (cetaceans) species. Among artiodactyls, the hippopotamuses are the only species adapted to an amphibious lifestyle. Moreover, recent morphological and molecular evidence suggests that modern hippopotamuses are the closest extant relatives of cetaceans (Gatesy, 1997; Shimamura et al., 1997; Milinkovitch, 1998; Nikaido et al., 1999; Agnarsson and May-Collado, 2006; Spaulding et al., 2009; Zhou et al., 2011).

Comparing closely related species (i.e., hippopotamuses and cetaceans) is particularly important insofar as differences in brain structure may account for differences in behavior. Although brain structures tend to be more similar in close relatives than in distantly-related species, environmental variables (e.g., whether an animal is terrestrial, amphibious, or fully aquatic) are important determinants for brain structure and function. In this regard, the brain of other marine mammals (e.g., manatees and dugongs, and pinnipeds, polar bears, and otters) could certainly help elucidate which neuroanatomical features might be linked to the aquatic environment. Nevertheless, it would be difficult to differentiate between neural characteristics due to environmental adaptation from those due to phylogenetic differences. Consequently, hippopotamuses and cetaceans represent a key comparison point for the understanding of brain evolution during the transition, for a particular group of mammals (the cetaceans), from a terrestrial environment to an obligatory aquatic one. Their phylogenetic relatedness, the differences in brain size and social behavior, as well as the diverse niches they occupy, allow for exploration of the possible relationships between cortical structure, phylogenetic constraints, and adaptation to the aquatic environment.

Although it is relatively clear that cetaceans split from other artiodactyls around 55 million years ago, at the start of the radiation of archaeocetes (archaic cetaceans) that led to living whales and dolphins (Gingerich, 1998, 2005; Thewissen et al., 2007; Uhen, 2010), hippopotamids have a shorter fossil record of about 23 million years (Boisserie et al., 2011) and their evolutionary history is contentious. Several different extinct groups of early terrestrial or semi-aquatics artiodactyls, including anthracotheres and raoellids, have been suggested as hippopotamid ancestors (Boisserie, 2005; Spaulding et al., 2009; Boissière et al., 2011; O’Leary et al., 2012). Because of uncertainty about the exact closest fossil relatives for both hippopotamids and cetaceans, it is not clear whether their most recent common ancestor was terrestrial or semi-aquatic (Boisserie et al., 2011). The modern family Hippopotamidae includes only two extant species: the river or common hippopotamus (Hippopotamus amphibius) and the pygmy hippopotamus (Hexaprotodon liberiensis). Five subspecies of river hippopotamuses are currently recognized based on skull shape and proportions (H. amphibius amphibius, H. a. tschadensis, H. a. kiboko, H. a. constrictis, and H. a. capensis; Lydekker, 1915; Grubb, 1993; Eltringham, 1999) but whether these differ genetically or are the result of intraspecific variability in skull characters remains unclear (Lydekker, 1915; Eltringham, 1999). Two subspecies of pygmy hippopotamus have been recognized in the past, but the only remaining species is H. liberiensis liberiensis, which is restricted to riverine environments in Liberia, Guinea, Sierra Leone, and Ivory Coast (Corbet, 1969; Eltringham, 1999). The pygmy hippopotamus’ body size is six to eight times smaller than that of H. amphibius, with adult H. liberiensis having a body mass equivalent to a 6-month-old H. amphibius (Eltringham, 1999). The head of the pygmy hippopotamus is also proportionally smaller and narrower than that of the river hippopotamus and its orbits are not raised above the skull roof, a feature that has been linked to less-aquatic habits than its larger relative (Eltringham, 1993b).

The organization of the brain of cetaceans is well documented at the gross anatomical level (Marino et al. 2001a,b,c, 2002, 2003a,b, 2004a,b; Montie et al., 2007, 2008; Morgane et al. 1980; Hof and Van der Gucht, 2007; Oelschlager and Oelschlager, 2008), as well as the level of cyto- and chemoarchitecture (Jacobs et al. 1971, 1979, 1984; Morgane et al., 1982, 1988; Glezer and Morgane, 1990b; Glezer et al., 1992, 1993, 1998; Hof et al., 1999; Hof and Sherwood, 2005; Manger et al., 1998; Hof et al., 2005; Hof and Van der Gucht, 2007; Furutani, 2008). In contrast, very little is known about the organization of the brain of hippopotamuses apart from its gross anatomy (Garrod, 1880; Pilleri, 1962). Such a lack...
of information is probably due to both the restricted geographic distribution of the extant hippopotamuses within the African continent, and the small numbers of individuals comprising the worldwide population (estimated at 157,000 river hippopotamuses and a few thousand, at most, pygmy hippopotamuses; Eltringham, 1993a,b) that make it difficult to obtain brain specimens. This is particularly true for the pygmy hippopotamus given its “Endangered” status in the International Union for Conservation of Nature (IUCN, 2013) red list (IUCN Red List of Threatened Species, Version 2013.2).

Here we describe the brain of *H. liberiensis* using magnetic resonance imaging (MRI) and a variety of histological approaches, including Nissl and Golgi staining, and immunohistochemistry. Moreover, we provide volumetric measurements of salient structures from MR images, as well as the values for the gyrencephalic index (GI), the cerebellar quotient (CQ), and the cerebellar index (CI). Finally, we report new findings on the distribution of von Economo neurons (VENs) first observed in this species in the insular cortex (Butti and Hof, 2010). Comparisons with cetaceans and other artiodactyls are made, when possible, to elucidate the evolutionary significance of certain neuroanatomical features.

### MATERIALS AND METHODS

#### Specimen and Histological Preparation

The brain of a 33-year-old female pygmy hippopotamus (brain mass: 262 g; body mass: 284 kg; Fig. 1A,B) that died of natural causes at the Cleveland Metroparks Zoo was collected during necropsy, 4 hr after death, and postfixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS). The brain was then hemisected. After MR scanning (see the Magnetic Resonance Imaging section for details), the right hemisphere was cut into serial slabs, cryoprotected in graded sucrose solutions up to 30% in PBS, frozen in dry ice, and processed serially into 80-μm thick sections on a sliding microtome (Leica Biosystems, Nussloch, Germany). Every 10th section was mounted on glass slides, stained with a solution of 0.2% cresyl violet, and coverslipped using DPX for examination. An additional adjacent series of sections was used for immunohistochemistry (see the Immunohistochemistry section for details). Separate blocks including the frontal magnocellular cortex, the cortex of the lateral gyrus and the anterior cingulate cortex (ACC) were sampled from the left hemisphere and stained with a modified rapid Golgi impregnation method (see the Golgi Staining section for details). All photomicrographs were obtained on a Zeiss Axioshot photomicroscope equipped with Plan-Neofluar objectives 2.5× (N.A. = 0.075), 40× (N.A. = 0.75), Plan-Apocromat objectives 10× (N.A. = 0.32) and 20× (N.A. = 0.8) (Zeiss, Thornwood, NY), and an Optronics MicroFire digital camera (Optronics, Goleta, CA). Images were adjusted for luminosity and contrast using Adobe Photoshop and drawings were made using Adobe Illustrator.

#### Immunohistochemistry

Sections were washed in phosphate buffer saline (PBS) and treated for antigen retrieval in a solution of citrate buffer (pH 8.0–9.0) in a 95°C water bath for 10 min. After cooling for 20 min at room temperature, the sections were rinsed in PBS and treated for 20 min in a solution of 0.3% hydrogen peroxide in PBS to eliminate endogenous peroxidase activity. After washing in PBS containing 0.3% Triton-X100, the blocking step was performed for 65 minutes in a solution of 5% normal goat serum, 2% bovine serum albumin, and 0.2% cold water fish gelatin in PBS containing 0.3% Triton-X100. The sections were then incubated overnight, at room temperature on a shaker, in a solution of the primary monoclonal mouse anti-calretinin (CR) antibody (Swant, Bellinzona, Switzerland; dilution 1:6,750; Schwaller et al., 1993) diluted in PBS containing 0.3% Triton-X100. Sections were then washed in PBS containing 0.3% Triton-X100 and incubated in a solution of the secondary polyclonal goat anti-mouse biotinylated antibody (Dako, Glostrup, Denmark; dilution 1:400) for 60 min. After washes in PBS containing 0.3% Triton-X100 the sections were washed in PBS containing 0.3% Triton-X100 and incubated in a solution of the secondary polyclonal goat anti-mouse biotinylated antibody (Dako, Glostrup, Denmark; dilution 1:400) for 60 min. After washes in PBS containing 0.3% Triton-X100 the sections were washed in PBS containing 0.3% Triton-X100 and incubated in a solution of the secondary polyclonal goat anti-mouse biotinylated antibody (Dako, Glostrup, Denmark; dilution 1:400) for 60 min. After washes in PBS containing 0.3% Triton-X100 the sections were washed in PBS containing 0.3% Triton-X100 and incubated in a solution of the secondary polyclonal goat anti-mouse biotinylated antibody (Dako, Glostrup, Denmark; dilution 1:400) for 60 min. After washes in PBS containing 0.3% Triton-X100 the sections were washed in PBS containing 0.3% Triton-X100 and incubated in a solution of the secondary polyclonal goat anti-mouse biotinylated antibody (Dako, Glostrup, Denmark; dilution 1:400) for 60 min.
were processed with the avidin-biotin peroxidase method using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for 60 min. The 3,3-diaminobenzidine (DAB) peroxidase kit (Vector Laboratories, Burlingame, CA) was then used as a chromogen to visualize the product of the reaction. The immunoreactivity was intensified using nickel. After washes in a solution of 50% PBS and 50% dH2O, the sections were mounted on glass slides, dehydrated in graded alcohol solutions up to 100%, immersed in a clarifying agent (limonene) and coverslipped using 100% DPX for examination.

The monoclonal anti-CR antibody was produced in mice by immunization with recombinant human CR 22 kD (Schwaller et al., 1993; Zimmermann and Schwaller, 2002) and reacts specifically with CR in tissue from human and rat with no cross-reaction with other calcium-binding proteins (Swant Monoclonal anti-CR 683 product description). Immunoreactivity to CR is observed mostly in non-pyramidal neurons and is colocalized with the major inhibitory neurotransmitter γ-aminobutyric acid (GABA). Thus, the CR-immunoreactive neurons represent inhibitory interneurons (Rogers, 1992; Andressen et al., 1993; DeFelipe, 1993; Condé et al., 1994; Kubota et al., 1994; DeFelipe, 1997; Gonchar and Burkhhalter, 1997). Although this CR-antibody reveals specific reactivity with CR in tissue from human and rat, the highly conserved sequence of the CR protein in evolution, from bacteria to mammals (Parmentier and Lefort, 1991), makes it possible to demonstrate immunostaining specificity of this antibody with interneurons in many other species including birds, reptiles, and mammals (Rogers, 1989; Baimbridge et al., 1992; Martinez-Guijarro and Freund, 1992; Résumois and Rogers, 1992; Glezer et al., 1993, 1998; Hof et al., 1996a,b, 1999; Davila et al., 1997; DeFelipe, 1997; Sherwood et al., 2009). This antibody has been previously used to study GABAergic interneurons of cetaceans and artiodactyls (Glezer et al., 1993, 1998; Hof et al., 1999), revealing staining patterns consistent with those observed in other mammalian species.

Golgi Staining

Tissue blocks from the frontal magnocellular cortex (likely corresponding to motor cortex), from the cortex of the lateral gyrus (likely corresponding to the visual cortex), and from the ACC, were removed from the left hemisphere, trimmed to 3 to 5 mm in thickness, and processed by a modified rapid Golgi technique (Scheibel and Scheibel, 1978). Briefly, after being immersed in a 0.33% osmic acid solution in the absence of light for 72 hr at room temperature, the osmicated tissue was placed in a 0.75% silver nitrate solution for 24 hr. The blocks were then cut into 120-μm thick sections using a vibratome (752M Vibroslice Campden Instruments, IN) and coverslipped with Permount for examination. Tracings of neurons in three dimensions, as previously done by the authors in the African elephant (Loxodonta africana; Jacobs et al., 2011), could not be performed because relatively poor impregnation prohibited detailed tracing of dendrites in their entirety. However, the Golgi-impregnated materials allowed for a qualitative description of neuronal morphologies based on cortical and laminar localization, soma size, and dendritic arbor characteristics.

Magnetic Resonance Imaging

MRI data were obtained using a 3 T Siemens Allegra MRI system at the Icahn School of Medicine at Mount Sinai. A high-resolution T2-weighted anatomical volume of the whole brain was acquired with a turbo spin echo pulse sequence. The imaging protocol was as follows: TR (repetition time) = 6,300 ms, TE (echo time) = 75 ms, number of signal averages = 10, slice thickness = 2 mm, gap = 0, matrix size = 256 × 256, and FOV (field of view) = 17.9 cm. Raw DICOM images were converted to NIFTI files using the statistical parametric mapping package (SPM5; Wellcome Department of Imaging Neuroscience, London, UK). These were then entered into Christopher Rorden’s MRIcron program (http://www.cabiatl.com/mricro/micro/index.html) to generate the final images for display.

Volume and Area Measurements

Manual image segmentation of MRIs was performed on processed coronal T2-weighted images to define the volume of the whole brain and of the cerebellum, hippocampus, and corpus callosum. We have chosen to report the volume of the corpus callosum, although most studies report only its midsagittal cross-sectional area, as the volume obtained by means of MRI represents a more accurate quantitative measure. Such values can be useful for future comparisons, as MR imaging becomes more widespread in comparative neuroanatomy. The manual segmentation of the whole brain, cerebellum, hippocampus, and corpus callosum was performed using ImageJ (Version 1.45s, NIH). Matlab (Version 2012a, Mathworks Inc., Natick, MA) code was developed to import the regions of interest (ROI) into the in-house software of the Department of Radiology, Icahn School of Medicine at Mount Sinai, and the volumes were computed. The area of the ROIs in each MRI slice was directly obtained by manual segmentation using the in-house software. Volumes were then calculated by integrating the selected area of each section. Every section containing the ROIs was used in the segmentation process. The manual segmentation of each ROI was performed three times and the mean volume was taken as a final value. The percentage of total brain occupied by each ROI was then calculated by dividing the volume of each ROI by the volume of the whole brain multiplied by 100 (Table 1).

The midsagittal area of the corpus callosum (CCA) was obtained by manually tracing the callosal perimeter at the midline level in a photograph (Fig. 1C) using ImageJ. This step was necessary, as the quality of the MR images did not allow for a sagittal view of the scan with an optimal resolution. The callosal perimeter was traced three times on the same photograph and the mean area was taken as a final value (Table 2). Finally, the mid-sagittal area of the corpus callosum/brain mass ratio (CCA/BM) was calculated both as reported by Tarp-ley and Ridgway (2000), dividing the cross-sectional area of the corpus callosum (in mm²) by the brain mass (in g), and as reported by Manger et al. (2010), dividing the square root of CCA (in mm²) by the cubic root of brain mass (in g) to account for effects of differing units of measure (Table 3). We have chosen to calculate the CCA/BM ratio using both methodologies, as the
above-mentioned studies report values for a series of different species relevant to our study. Using both values facilitates comparisons of our results with previously published data (Tables 2 and 3).

Gyrencephalic Index, Cerebellar Quotient, and Cerebellar Index

The gyrencephalic index (GI) is a measure of the folding of the cerebral cortex calculated by dividing the perimeter of the pial contour of the cortex by the perimeter of its outer contour (Zilles et al., 1989). The pial contours and the outer contours of 41 coronal sections throughout the brain of the pygmy hippopotamus were measured using the NIH software ImageJ. The GI was calculated by dividing the sum of the 41 pial contours by the sum of the 41 outer contours. The tracing of the brain sections to define pial and outer contours was performed three times by the same operator (C.B.) and the average value of the three measurements was taken as a final value of GI (Table 4).

The cerebellar quotient (CQ) was calculated using the equation provided by Maseko et al. (2010) (Table 5). The cerebellar index was calculated by dividing the cerebellar volume by the whole brain volume as measured in the MRI. Moreover, in the present study we have calculated values of CQ and cerebellar index (CI) for some relevant species of artiodactyls, marine mammals other than cetaceans, and eutherian mammals of comparable brain size for which brain mass, vermal volume and hemispheric volume were reported in Maseko et al. (2010) (Table 5). Particularly, vermal and hemispheric volumes, reported by Maseko and colleagues, were summed to obtain an approximation of cerebellar volumes. Given that our calculation excluding the cerebellar peduncles in the three specimens of elephants resulted in the same value of cerebellar volume reported by Maseko et al. (2010) for these specimens, we considered our approximation sufficiently robust to estimate the total cerebellar volume in relevant species of artiodactyls and carnivores for which hemisphere volume and vermal volume were available. Brain volumes were obtained by multiplying brain mass by the specific mass of brain tissue (1.036). CQ and CI were then calculated as indicated above.

RESULTS

General Morphology

We utilized the nomenclature available for cetaceans and artiodactyls for the identification of gyri and sulci (Gruenberger, 1970; Morgane et al., 1980; Pilleri, 1980; Hof and Van der Gucht, 2007). This nomenclature is applicable with variations to all species presenting a more or less verticalized (pseudo) Sylvian cleft, as it is the case in the pygmy hippopotamus. In fact this nomenclature has been applied in practice, with variants, across a broad number of species from different orders and extends to carnivores as well as perissodactyls.

The whole fixed brain of the pygmy hippopotamus weighed 262 g and measured 13.5 cm in its anteroposterior axis (Fig. 1A,B). The medial aspect of the hemisphere was characterized by three main well-defined sulci: rostroventrally, the olfactory sulcus, that divided the frontal gyrus from the gyrus rectus; dorsal to the...
corpus callosum, the cingulate sulcus, which began at a level corresponding to the genu of the corpus callosum and ran towards the most caudal part of the brain, defining the cortical belt that lies dorsal to the corpus callosum (namely, the cingulate gyrus) and separating it from the frontal and parietal gyri; and caudally, the smaller (and almost vertical) calcarine sulcus that divided the parasplenial gyrus from the lingual gyrus (Fig. 1B).

The dorsal aspect of the hemisphere was characterized by the lateral gyrus, which was delimited medially by the paracingulate sulcus and laterally by the ectolateral sulcus. The lateral aspect of the hemisphere was characterized by three gyri: the large suprasylvian gyrus, the diminutive ectosylvian gyrus, and the perisylvian gyrus (Fig. 1A). Similar to cetaceans, these three gyri were concentrically organized around a pronounced and almost vertical Sylvian fissure that divided the Sylvian cortex into anterior and posterior limbs (Gruenberger, 1970; Morgane et al., 1980; Pilleri, 1980; Hof and Van der Gucht, 2007). Rostrally was a well-defined cruciate sulcus that divided the coronal gyrus from the sigmoid gyrus, and an orbital sulcus, which divided the anterior limb of the Sylvian cortex from the more rostral orbital and frontal gyri (Fig. 1A). Finally, a large olfactory bulb originated ventrally to the gyrus proreus (Fig. 1A,B).

MRI, Volumetric Measurements, CCA/BM Ratio, GI, CQ, and CI

Figure 2 shows a sequence of 2-mm thick MR coronal images originally acquired at an interval of 12 mm. MRI images show that a portion of the basal forebrain, ventral striatum, hypothalamus and brainstem was missing in the left hemisphere following brain removal from the skull. Volumetric measurements performed on this series of MR images showed that the corpus callosum measured 1.62 cm³ and occupied about 0.58% of the total brain volume. The hippocampus measured 8.53 cm³ (left hippocampus = 3.87 cm³ and right hippocampus = 4.66 cm³; Table 1), occupied 3.07% of the total brain volume and possessed well-developed components (dentate gyrus

### TABLE 2. Brain mass (BM), midsagittal area of the corpus callosum (CCA), and CCA/BM ratio in the pygmy hippopotamus and comparison with available data in marine mammals

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>Mean BM (g)</th>
<th>Mean CCA (mm²)</th>
<th>CCA/BM</th>
</tr>
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<tr>
<td>Hippopotamidae</td>
<td><em>Hexaprotodon liberiensis</em></td>
<td>262a</td>
<td>193a</td>
<td>0.737a</td>
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<td>Delphinidae</td>
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<td><em>Steno bredanensis</em></td>
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<td>643.28b,d</td>
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<td>0.829a</td>
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aData based on a single specimen.
bData from Tarpley and Ridgway (1994).
cData from Montie et al. (2008).
dNeonate.

### TABLE 3. Brain mass (BM), midsagittal area of the corpus callosum (CCA), and CCA/BM ratio (calculated as in Manger et al., 2010) in the pygmy hippopotamus and comparison with available data in relevant eutherian mammals

<table>
<thead>
<tr>
<th>Family</th>
<th>BM (g)</th>
<th>CCA (mm²)</th>
<th>CCA/BM</th>
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<td>262</td>
<td>193</td>
<td>0.22</td>
</tr>
<tr>
<td>Cetaceans</td>
<td>630–6,060</td>
<td>139.3–565.3</td>
<td>0.11–0.15</td>
</tr>
<tr>
<td>Sirenians</td>
<td>188</td>
<td>89.5</td>
<td>0.17</td>
</tr>
<tr>
<td>Pinnipeds</td>
<td>345–1,250</td>
<td>101–188.7</td>
<td>0.13–0.17</td>
</tr>
<tr>
<td>Artiodactyls</td>
<td>244–530</td>
<td>117.3–180.1</td>
<td>0.16–0.18</td>
</tr>
<tr>
<td>Perissodactyls</td>
<td>531–585</td>
<td>238.5–251.5</td>
<td>0.19–0.20</td>
</tr>
<tr>
<td>Carnivores</td>
<td>8.30–470</td>
<td>7.2–237.0</td>
<td>0.13–0.21</td>
</tr>
</tbody>
</table>

aData based on a single specimen.
bPresent study.
cData from Manger et al. (2010).
TABLE 4. Brain mass (g), cerebellar volume (cm³), brain volume (cm³), cerebellar quotient (CQ) and cerebellar index (CI) in the pygmy hippopotamus and relevant species of artiodactyls, cetaceans, and carnivores

<table>
<thead>
<tr>
<th>Species</th>
<th>Brain mass (g)</th>
<th>GI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetartiodactyla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H. liberiensis</td>
<td>262</td>
<td>1.84</td>
</tr>
<tr>
<td>Sus scrofa b</td>
<td>95.3</td>
<td>2.16</td>
</tr>
<tr>
<td>Odocoileus virginianus b</td>
<td>160</td>
<td>2.27</td>
</tr>
<tr>
<td>Lama glama b</td>
<td>200.3</td>
<td>2.70</td>
</tr>
<tr>
<td>Bos taurus indicus b</td>
<td>474</td>
<td>2.53</td>
</tr>
<tr>
<td>Kogia simus b</td>
<td>577</td>
<td>5.26</td>
</tr>
<tr>
<td>Delphinus delphis b</td>
<td>981</td>
<td>5.41</td>
</tr>
<tr>
<td>Tursiops truncatus b</td>
<td>1,530</td>
<td>5.63</td>
</tr>
<tr>
<td>Delphinapterus leucas b</td>
<td>1,871</td>
<td>5.23</td>
</tr>
<tr>
<td>Orcinus orca b</td>
<td>5,617</td>
<td>5.70</td>
</tr>
<tr>
<td>Megaptera novaeangliae b</td>
<td>4,600</td>
<td>5.35</td>
</tr>
<tr>
<td>Carnivores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phoca vitulina b</td>
<td>275</td>
<td>2.38</td>
</tr>
<tr>
<td>Zalophus californianus b</td>
<td>379.1</td>
<td>2.52</td>
</tr>
<tr>
<td>Panthera leo a</td>
<td>258</td>
<td>1.85</td>
</tr>
</tbody>
</table>

aPresent study.
bData from Manger et al. (2012).

and CA fields) (Fig. 2). The cerebellum measured 26.95 cm³ and occupied 9.72% of the total brain volume.

The CCA/BM ratio, as calculated as reported by Tarpaley and Ridgway (2000), was 0.737. However, when the CCA/BM ratio was calculated correcting for the effect of differing units of measure (Manger et al., 2010), the value obtained was 0.22 (Fig. 2, Tables 1 and 2). Table 3 provides comparison points for these measures. The average value of three repeated measurements of GI resulted equal to 1.84. The cerebellar quotient and the cerebellar index resulted equal to 1.84. The cerebellar quotient and the average value of three repeated measurements of GI provides comparison points for these measures. The value obtained was 0.22 (Fig. 2, Tables 1 and 2). Table 3 differing units of measure (Manger et al., 2010), the CCA/BM ratio was calculated correcting for the effect of

<table>
<thead>
<tr>
<th>Species</th>
<th>Brain mass (g)</th>
<th>GI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnivores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phoca vitulina b</td>
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<td>2.38</td>
</tr>
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<td>Zalophus californianus b</td>
<td>379.1</td>
<td>2.52</td>
</tr>
<tr>
<td>Panthera leo a</td>
<td>258</td>
<td>1.85</td>
</tr>
</tbody>
</table>

**Cortical Organization**

The cortical organization of the gyri bounded by the major sulci is identified in Fig. 1. The cytoarchitecture of cortical areas was defined according to topographical location. Based on coronal Nissl-stained sections, the neocortex of the pygmy hippopotamus was entirely agranular—with no identifiable layer IV. Consequently, we do not mention the absence of layer IV further.

**Paleocortex**

The paleocortex includes the olfactory cortex, the olfactory tubercle, and the piriform cortex. The piriform cortex, which abuts the anterior insular cortex, represents the most anterior portion of the paleocortex and it was characterized by a thick layer I, a thick and dense layer II, and a layer III containing sparse and small pyramidal neurons (Fig. 3A). Ventral to the piriform cortex, the cortex of the olfactory tubercle was much thinner and was characterized by clusters of neurons in layer II and a poorly defined transition between layers II and III (Fig. 3B). Posteriorly, the periamygdalar cortex, which abuts the archi- and entorhinal cortices, was characterized by clusters of small neurons in its most superficial layers and by a visible lamina dissecans (Fig. 3C).

**Archicortex**

The hippocampus of the pygmy hippopotamus appeared comparable to that of other terrestrial mammals (Fig. 4A). The dentate gyrus was large and contained a thick and hyperchromatic granular layer underlined by a very thick subgranular zone, the polymorphic layer (Fig. 4A,B). Below the polymorphic layer of the dentate gyrus, the hilar part of CA3 was thick (Fig. 4A,B) and extended laterally into a long CA3 field that contained a thick and loosely organized pyramidal layer (Fig. 4C). CA2 was clearly visible as a region where the pyramidal layer becomes disorganized and its neurons are very sparse before the transition into the thin and well-organized pyramidal layer of CA1 (Fig. 4A,D). The pyramidal neurons of CA2 were comparable in size to those of CA3 and larger than those observed in CA1. CA1 was very well defined and contained a pyramidal layer that was organized in two sublayers: a dense and intensely stained thin superficial layer and a thick and less densely packed lower layer containing pyramidal neurons of smaller size than those of the superficial sublayer (Fig. 4D). The transition of CA1 to the subiculum (Fig. 4A,E) was clearly visible as the pyramidal layer becomes thicker and homogeneous, lacking the subdivision in two sublayers observed in CA1. Laterally, the small presubiculum abutted the parasubiculum, which displayed a thick superficial pyramidal layer that contained small and darkly labeled pyramidal neurons, a thin lamina dissecans, and a deeper pyramidal layer consisting on small neurons (Fig. 4F).

**Entorhinal Cortex**

The entorhinal cortex of the pygmy hippopotamus, unlike in cetaceans but similar to terrestrial mammals, appeared to be proportionate to the size of the dentate gyrus. Anteriorly, the entorhinal cortex was thin with a prominent clustering of layer II neurons in large and darkly stained islands, a very thick layer III containing medium size neurons, a broad lamina dissecans, a thin layer V with an irregular distribution of medium size neurons, and a multiform layer VI (Fig. 4G). Moving laterally (dorsally) the overall neuronal density decreased, the layer II islands tended to disappear and the lamina dissecans became thinner than in the most medial portion of this cortex (Fig. 4H). Posteriorly, the caudal entorhinal cortex presented an overall increased neuronal density compared with more anterior levels, particularly in layers III, V, and VI (Fig. 4I). Layer II still showed some clustering but the islands were larger and the neurons were less tightly packed than in anterior levels (Fig. 4I). The lamina dissecans was thinner than in the anterior sector, but was still clearly visible. In contrast, layers V and VI were much thicker (Fig. 4I). Laterally, at the caudal level, the transition of the entorhinal cortex into the temporal cortex was marked by the disappearance of the lamina dissecans, the transition of clustered layer II into a homogeneous and thinner layer of neurons, and by the disappearance of a marked transition between layer V and VI.
Cingulate and Retrosplenial Cortex

The cingulate cortex consisted of one gyrus outlined ventrally by the callosal sulcus and dorsally by the cingulate sulcus (Fig. 1). The cingulate and retrosplenial cortices were divided into a subgenual area, a supracallosal area including the pregenual, anterior and posterior cingulate cortices, and the retrospl enial cortex posterior to the splenium of the corpus callosum, including an anterior and a posterior component, recognizable by their different cortical organization. In coronal sections an additional small sulcus, ventral to the cingulate sulcus and limited to the anterior portion of the cingulate cortex, could be appreciated. The banks of these sulci were linear and lacked the convolution often observed in cetaceans (Morgane et al., 1982; Hof and Van der Gucht, 2007).

Subgenual Cortex

The subgenual cortex is the portion of the anterior cingulate cortex that lies ventral to the genu of the corpus callosum. It was characterized by a layer II that contained numerous small and irregular neuronal clusters (Fig. 5A). These clusters were also observed, to a lesser degree, in the adjacent pregenual cortex (Fig. 5B), unlike in cetaceans where clustering of layer II is not present in the pregenual cortex (Hof and Van der Gucht, 2007). Layer III contained medium-size neurons and its transition to layer V was not well defined. Layer V was thick, contained medium-sized neurons. Its boundary with layer VI was not easily identifiable. Layer VI was thin and populated by small neurons (Fig. 5A).

Pregenual Cortex

The pregenual cortex is the portion of cortex situated directly in front of the genu of the corpus callosum. It presented, below a thick layer I, a layer II that was more homogeneous than layer II of the adjacent subgenual cortex and showed an irregular pattern of clustering. Layer III was thick and populated by small neurons. Layer V was thick and characterized by larger neurons than in the adjacent subgenual cortex. Layer VI was thin and contained small neurons with an occasional columnar arrangement (Fig. 5B).

Anterior and Posterior Cingulate Cortex

At a level corresponding to the genu of the corpus callosum, the anterior cingulate cortex (ACC) was characterized, in its ventral bank, below a thick layer I, by a layer II of variable thickness, a thick layer III containing small relatively sparse neurons, a well-developed layer V consisting of large pyramidal neurons, and a thick, multiform layer VI (Fig. 5C). The dorsal bank of the ACC, at the same level, was characterized by a homogeneous and dense layer II, a layer III containing small size pyramidal neurons, a layer V containing large pyramidal neurons in large clusters that occasionally overlapped with the underlying multiform layer VI (Fig. 5D).

The posterior cingulate cortex (PCC) showed several variations from the pattern described in the ACC. In the PCC, in fact, the clustering of layer II was not present. Layer V thickened in the ventral portion of the PCC, in comparison to the ventral aspect of the ACC whereas, in the dorsal aspect of the PCC, layer V contained large pyramidal neurons organized in vertical modules that tended to overlap with the neurons of layer VI (Fig. 5E,F).

Anterior and Posterior Retrosplenial Cortex

The retrospl enial cortex showed, in both its anterior and posterior sectors, an overall increased neuronal density and a thinner and more homogeneous layer II than the ACC and PCC (Fig. 5G,H). The anterior sector of the retrosplenial cortex possessed a thick layer III populated by neurons that were comparable in size to those observed in layer III of the ACC (Figs 5C,G). Layer V was variable in thickness; it contained large pyramidal neurons and lacked the clustering of layer V observed in the dorsal portion of the ACC (Fig. 5D,G). Layer VI was thick and contained small neurons. The posterior sector of the retrospl enial cortex contained a diminutive layer III,

### TABLE 5. Brain mass (g) and gyrencephalic index (GI) in the pygmy hippopotamus and comparison with artiodactyls, cetaceans, and relevant species of other eutherian mammals

<table>
<thead>
<tr>
<th>Species</th>
<th>Brain mass (g)</th>
<th>Cerebellar volume (cm³)</th>
<th>Brain volume (cm³)</th>
<th>CQ</th>
<th>CI*10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetartiodactyls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. liberiensis</em>[^a^]</td>
<td>262</td>
<td>26.95</td>
<td>277.32</td>
<td>0.80</td>
<td>0.97</td>
</tr>
<tr>
<td><em>Sus scrofa</em>[^a^]</td>
<td>96.30</td>
<td>9.28</td>
<td>98.73</td>
<td>0.74</td>
<td>0.94</td>
</tr>
<tr>
<td><em>Odocoileus virginianus</em>[^a^]</td>
<td>160</td>
<td>18.75</td>
<td>165.76</td>
<td>0.90</td>
<td>1.13</td>
</tr>
<tr>
<td><em>Llama glama</em>[^b^]</td>
<td>200.30</td>
<td>22.46</td>
<td>207.51</td>
<td>0.87</td>
<td>1.08</td>
</tr>
<tr>
<td><em>Bos taurus indicus</em>[^b^]</td>
<td>520.50</td>
<td>33.71</td>
<td>539.24</td>
<td>0.51</td>
<td>0.62</td>
</tr>
<tr>
<td><em>Phocoena phocoena</em>[^b^]</td>
<td>486</td>
<td>66.84</td>
<td>503.5</td>
<td>1.08</td>
<td>1.33</td>
</tr>
<tr>
<td><em>Phocoena phocoena</em>[^b^]</td>
<td>503</td>
<td>74.08</td>
<td>521.10</td>
<td>1.16</td>
<td>1.42</td>
</tr>
<tr>
<td><em>Tursiops truncatus</em>[^b^]</td>
<td>1,500</td>
<td>258.75</td>
<td>1,554</td>
<td>1.40</td>
<td>1.66</td>
</tr>
<tr>
<td>Carnivores</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phoca vitulina</em>[^b^]</td>
<td>275</td>
<td>24.01</td>
<td>284.9</td>
<td>0.70</td>
<td>0.84</td>
</tr>
<tr>
<td><em>Zalophus californianus</em>[^b^]</td>
<td>379.13</td>
<td>58.19</td>
<td>392.78</td>
<td>1.20</td>
<td>1.48</td>
</tr>
<tr>
<td><em>Ursus maritimus</em>[^b^]</td>
<td>458.60</td>
<td>51.95</td>
<td>475.11</td>
<td>0.90</td>
<td>1.09</td>
</tr>
<tr>
<td><em>Panthera leo</em>[^b^]</td>
<td>258</td>
<td>26.54</td>
<td>267.3</td>
<td>0.80</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Cerebellar volume was calculated adding the vermal volume multiplying brain mass by the specific mass of nervous tissue and by the gyrencephalic index (GI) as indicated in the Materials and Methods section.

[^a^] Present study.
[^b^] From Manger et al. (2012).
which was characterized by small-size neurons. The large pyramidal neurons of layer V possessed a vertical organization that, however, did not continue into layer VI as observed in the dorsal aspect of the PCC (Fig. 5H,F). Layer VI was thick and contained medium-size neurons.

Insular Cortex

The insular cortex lies in the depth of the Sylvian fissure. In H. liberiensis the insula was operculated by parts of the frontal, parietal, and temporal cortices. Its extent in this species it was limited only to a few gyri (Butti and Hof, 2010).

Anterior Insular Cortex

The anterior portion of the insular cortex was limited dorsally by what may correspond to the primary somatosensory cortex (SS; see below), and ventrally by the piri- form cortex. In the most dorsal portion of the anterior insular cortex layer II was thin, contained medium size neurons, and exhibited only a weak clustering of neurons. Layer III was very thick and contained small- and
medium-sized neurons. Layer V was thick and populated by medium-sized and occasionally large pyramidal neurons. Layer VI was thick and contained small neurons. Small islands of claustral neurons were present in the underlying white matter (Fig. 6A).

In the depth of the Sylvian fissure, the cortex became thinner overall. Layer II was more homogeneous and its neurons were sparser than dorsally. Layer V was thin and contained neurons comparable in size to those observed in the most ventral portion of the anterior insular cortex (Fig. 6B). At this level, layers V and VI were characterized by a columnar appearance with large and elongated claustral islands in the underlying white matter (Fig. 6B). In its most ventral aspect, the anterior insular cortex exhibited a layer II characterized by an irregular distribution of neurons, a very thick layer III containing small-size neurons, and by a reduced thickness of layer V compared with the dorsal aspect of the region (Fig. 6C). Layer V contained occasional groupings of neurons and layer VI was very thin. Claustral islands became larger where, ventrolaterally, the insular cortex abutted the piriform cortex (Fig. 6C).

**Mid and Posterior Insular Cortex**

The middle and posterior portions of the insular cortex were limited dorsally by what appears to correspond to the auditory cortex (see Parietal Cortex for details) and ventrally by the piriform cortex. In its most dorsal aspect, the midinsular cortex showed a much thinner, less dense and less homogeneous layer II than observed in the anterior insular cortex. Layer III was very thick and contained small-sized neurons. Given the large difference in neuronal size between layer III and layer V, the transition between these two cortical layers was well defined in the middle portion of the insular cortex (Fig. 6D–F). Layer V was organized in columnar structures of several neurons that formed a continuum with layer VI.

![Fig. 3. Cytoarchitecture of the paleocortex in the pygmy hippopotamus. Piriform cortex (A), cortex of the olfactory tubercle (B), and periamygdalar cortex. Coronal sections in rostro-caudal order showing the location of A–C (D, E). Layers are indicated by Roman numerals; Id, lamina dissecans. Scale bar = 400 μm (A–C), and 5 cm (D, E).](image-url)
Fig. 4. Cytoarchitecture of the archicortex, and entorhinal cortex in the pygmy hippopotamus. Panoramic view of the hippocampal formation (A), dentate gyrus (B), CA3 (C), CA1 (D), subiculum (E), presubiculum (F), anterior (G, H), and posterior entorhinal cortex (I). Note the well-developed dentate gyrus including a thick subgranular zone (A, B), CA3 (C), the thick pyramidal layer of CA1 (D), the thick subiculum (E), and presubiculum (F) with an evident lamina dissecans. The entorhinal cortex presents both rostrally (G, H) and caudally (I) a clear lamina dissecans, whereas the parvocellular islands of layer II become more evident caudally. Coronal sections in rostro-caudal order showing the location of panels B–I (J–L). Layers are indicated by Roman numerals. CA1–3, Ammon’s horn fields; DG, dentate gyrus; gl, granular layer; ld, lamina dissecans; ml, molecular layer; pl, polymorphic layer; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; SUB, subiculum, wm, white matter. Scale bar = 400 μm (B–I), 1.6 mm (A), and 5 cm (J–L).

(Fig. 6D), similar to the cortex lying in the depth of the Sylvian fissure in the anterior part of the insula (Fig. 6B). In the depth of the Sylvian fissure, the midinsular cortex exhibited a pattern of cortical organization comparable to that in the depth of the Sylvian fissure anteriorly (Fig. 6B,E). Ventrally the midinsular cortex was characterized by an increased density of layer II containing medium-size neurons, by a layer V with a variable thickness, and by a thick layer VI with a columnar arrangement of neurons. Large claustral islands were present in the underlying white matter (Fig. 6F).

Posteriorly, the organization of the insular cortex included a homogeneous layer II that was thicker than in the anterior and middle sectors of the insula, a thick layer III including small and medium-sized neurons (Fig. 6G,H) and a columnar organization of layers V and VI that was more evident ventrally (Fig. 6H) than dorsally (Fig. 6G). Claustral islands were evident at this posterior level along both the dorsal and the ventral aspect of the insular cortex (Fig. 6G–H).

Frontopolar Cortex and Frontal Convexity

The frontopolar region of H. liberensis was well defined and separated from the orbital cortex and the gyrus proreus by two shallow sulci in the frontal tip of
the brain that ran almost perpendicular to each other, (Fig. 1). The orbital cortex, including the gyrus proreus, was defined dorsally by the cortex of the sigmoid gyrus and by the orbital sulcus, which divided it from the anterior limb of the Sylvian cortex, and ventrally by the gyrus proreus (Fig. 1).

The cytoarchitecture of the frontopolar cortex was characterized by a thick layer I, and a dense, clustered layer II with large spaces of neuropil between adjacent clusters. The depth of such neuropil spaces was limited to layer II and, occasionally, extended to the most superficial part of layer III. Layer III was thick and populated
by small neurons; layer V was extremely variable in thickness and contained larger pyramidal neurons than layer III. Layer VI was thick with a columnar organization (Fig. 7A). The peculiarity of this cortical region was the presence in layer II of clusters that varied greatly in size, thickness, and length (Fig. 7B–E).

The sigmoid gyrus was characterized by a thin magnocellular type of cortex. A well-defined and dense layer II, formed by small neurons, was followed by a thick layer III containing homogeneously distributed medium-size pyramidal neurons. Layer V consisted of large pyramidal neurons that were often grouped in clusters of several cells. Layer VI was thick and contained neurons that were arranged into large vertical modules directly aligned with the clusters of layer V (Fig. 7F). This cortical organization was observed from the midline to the most lateral extension of the sigmoid gyrus and continued dorsally until just below the cruciate sulcus. At this level the large pyramidal neurons of layer V progressively disappeared and layer II became heavily clustered. This magnocellular cortex is in a position to represent the putative motor cortex in the

Fig. 6. Cytoarchitecture of the insular cortex in the pygmy hippopotamus. Anterior dorsal (A), depth of Sylvian fissure (B), anterior ventral (C), middle dorsal (D), depth of Sylvian fissure (E), middle ventral (F), dorsal posterior (G), and ventral posterior (H) insular domains. Coronal sections in rostro-caudal order showing the location of panels A–H (I–K). Layers are indicated by Roman numerals. Scale bar = 400 μm (A–H), and 5 cm (I–K).
The pygmy hippopotamus and seemed to expand more laterally than rostrocaudally.

In the dorsal aspect of the sigmoid gyrus, just below the cruciate sulcus, the pattern of cortical organization changed. Here, the cortex was thicker, a marked clustering of layer II appeared, and the large neurons of layer V tended to disappear. Layer V was less well defined, and contained smaller neurons than the putative motor cortex. Moreover, neurons in the deep layers III and V were arranged in columns under the clusters of layer II. The spaces among the large columns were evident illustrating an extreme columnar appearance to the upper cortical layers. Layer VI was thick with neurons that seemed to conform to the columnar organization of the upper layers (Fig. 7G).

In the depth of the cruciate sulcus, the pattern of clustering of layer II was maintained, but the organization of the deeper cortical layers became more homogeneous (Fig. 7H). More dorsally in the coronal gyrus the layer II clusters decreased in size, layer III was thick and contained small neurons that were homogeneously distributed under the clusters of layer II. Layer V contained medium-sized neurons. Layer VI was thick with neurons organized in modules that created a columnar appearance (Fig. 7I). This parvocellular frontal field, which extended around the cruciate sulcus and dorsally to the

---

Fig. 7. Cytoarchitecture of the frontopolar cortex and the frontal convexity in the pygmy hippopotamus. (A–E) Frontopolar cortex, (F) magnocellular cortex of the sigmoid gyrus corresponding to the putative motor cortex, (G) cortex of the dorsal aspect of the sigmoid gyrus, just below the cruciate sulcus, (H) cortex in the depth of the cruciate sulcus, (I) cortex of the coronal gyrus. Note the clustering pattern of layer II (A–E; G–I) and the magnocellular elements in layer V of the putative motor cortex (F). Coronal sections in rostro-caudal order showing the location of panels A–I, (J–L). Layers are indicated by Roman numerals; wm, white matter. Scale bar = 400 μm (A–I); 5 cm (J–L).
magnocellular frontal field of the motor cortex, could represent the putative somatosensory cortex. This pattern of cortical organization extended laterally along the most rostral portion of the ectolateral sulcus and dorsally to the lateral gyrus for two thirds of its extent (see Parietal Cortex below for details).

Temporal Cortex

Although the temporal operculum was well defined in the pygmy hippopotamus, the concentric disposition of the small gyri and sulci around the Sylvian fissure can be misleading. For this reason we decided to consider temporal cortex, consistent with observations in cetaceans (Hof and Van der Gucht, 2007), as the portion of cortex situated below a horizontal line passing through the dorsal tip of the Sylvian fissure. Anteriorly and ventrally, the temporal cortex abutted the entorhinal cortex. At this level, the temporal cortex presented a thick cortical plate characterized by a prominent and thin layer II with occasional small clumps of neurons. The transition between deeper layers was not well defined at this level and the neuronal density was homogenous from layer III to VI. Layer V was thick and contained medium-size neurons (Fig. 8A).

Medially in the temporal pole the cortex exhibited a low neuronal density but a thick layer II that contained medium-size neurons. The transition to layer III was clearly visible given the smaller size of the neurons in this layer, which made it much less chromatic than the adjacent upper and lower layers. Layer V contained medium-size neurons distributed homogeneously and layer VI was thin with small neurons (Fig. 8B). Dorsally, at the tip of the temporal pole towards the parietal cortex, the cortex became thicker, with higher neuronal density. Layer V contained larger neurons than in the medial aspect and layer VI became thicker (Fig. 8C). Laterally, the cortex had reduced thickness, similar to the medial aspect. Layer V neurons appeared larger than dorsally and layer VI showed a progressive columnar organization (Fig. 8D). Moving caudally, the temporal cortex maintained the poorly defined transition between adjacent cortical layers, with the exception of layer II, which was extremely visible as its neuronal density increased and a clustering pattern became apparent, near the transition with the occipital cortex (Fig. 8E).

Parietal Cortex

The parietal cortex was a large domain located posteriorly to the coronal gyrus extending from the lateral gyrus, at the apex of the brain, down to the perisylvian gyrus and posteriorly delimited by the most caudal segment of the ectolateral sulcus (Fig. 1). At the apex of the brain, the first two-thirds of the lateral gyrus (just posterior to the coronal gyrus) were characterized by the same type of cortex observed in the dorsomost part of
the coronal gyrus. This appeared to be the putative somatosensory cortex. However, in the lateral gyrus, a rostrocaudal gradient of clustering of layer II was evident as the clusters became larger and thicker caudally (Fig. 9A–C). Moreover, an evident transition to a columnar arrangement of medium-sized neurons in layer VI was present, caudally, although the upper boundary of layer V was poorly defined (Fig. 9A).

In the pygmy hippopotamus the somatosensory type of cortex seemed to extend posteriorly along an extended portion of the lateral gyrus. It was only in the most posterior third of the lateral gyrus that we found a type of cortex with a thin and homogeneous layer II, a thick layer III containing small neurons, a layer V containing occasional large neurons, and a layer VI organized in modules (Fig. 9D). The pattern changed in the most medial aspect of the lateral gyrus where the modular organization of layer VI was evident and the small neurons of layer III become sparse (Fig. 9E). The cortical organization of the lateral aspect of the lateral gyrus expanded laterally into the suprasylvian gyrus and into the depth of the suprasylvian fissure. Such fields, in view of their position and cytoarchitecture, could correspond to the putative visual cortex; ectosylvian (F) and perisylvian cortex (G) showing the putative auditory cortex. Coronal sections in rostro-caudal order showing the location of panels A–G (H–K). Layers are indicated by Roman numerals; wm, white matter. Scale bar = 400 µm.
Laterally, in the ectosylvian cortex, the cytoarchitecture was characterized by a thin layer II, a high neuronal density in layer III, and by layers V and VI containing small neurons disposed in vertical modules (Fig. 9F). More laterally, layer II thickened into the perisylvian cortex. Layers V and VI were characterized by a lower cellularity than the ectosylvian cortex, but still displayed a high degree of columnar organization (Fig. 9G). These regions are in a position to represent auditory cortices as described in cetaceans (Sokolov et al., 1972; Popov and Supin, 1976, 1986; Ladygina and Supin, 1977; Ladygina et al., 1978).

**Occipital Cortex**

The occipital cortex is the posterior polar cortex and is situated posterior to the most caudal part of the ectolateral sulcus and includes the inferior portion of temporal cortex, which covers the anterior surface of the cerebellum. Most of the occipital cortex was characterized by clustering of layer II neurons. Medially, the occipital cortex was characterized by high neuronal density, variable thickness of layer II, large neurons in layer III, medium-sized neurons in a thick layer V, and a thick layer VI that displayed cellular columns (Fig. 10A). More dorsally the cortex was less dense and the layer II clusters were apparent. Layer II contained medium-sized neurons and could be divided into two separate sublayers, the most superficial arranged in clusters, and the deeper one being more homogeneous. Layer III was thick and contained sparse neurons. Layer V was formed by small-sized neurons and layer VI was organized in short columnar arrangements (Fig. 10B). Dorsally, towards the lateral aspect of the hemisphere, the organization of the cortex was reminiscent of the architecture of the putative visual cortex described above (Fig. 10C).

Laterally, the pattern of cortical organization changed. Layer II was organized into striking clusters, interspersed with large neuropil spaces. Layer III was thin and the very small size of its neurons contrasted with the size of neurons of layer II and V. Layer V was thick and formed by medium- to large-sized neurons, and layer VI was thin and contained medium-sized neurons (Fig. 10D). Ventrally, the clustering pattern of layer II disappeared gradually (Fig. 10E); however, occasional neuropil spaces interrupted the homogeneity of layer II, which was here divided in two sublayers, as in the dorso-lateral most aspect of the occipital cortex (see above and Fig. 10F). However, at this ventral level, the difference in density of the two sublayers was more evident due to

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**Fig. 10. Cytoarchitecture of the occipital cortex in the pygmy hippopotamus.** (A) medial; (B, C) dorsal and dorsolateral; (D) lateral, (E) ventral, (F) caudal aspects of the occipital cortex. Coronal sections in rostro-caudal order showing the location of panels A–F (G, H). Layers are indicated by Roman numerals; wm, white matter. Scale bar = 400 μm.
the intense staining of the superficial portion of layer II. Layer III was thin and characterized by very small neurons as observed in the lateral aspect of the occipital cortex (Fig. 10D,E). Layer V was thick and consisted of small neurons that often had an elongated morphology. Layer VI was thick and characterized by small-sized neurons. Clustering of layer II was maintained in the most caudal portion of the occipital cortex. However, the size of these clusters decreased and layer III was characterized by medium-sized neurons (Fig. 10F).

**Distribution and Morphology of Calretinin-Immunoreactive Neurons**

In the pygmy hippopotamus, CR-immunoreactive neurons were distributed homogeneously across cortical areas and displayed their highest densities in layers I, II, and superficial III, but were only sparsely visible in deeper layers (Fig. 11A–J). CR-immunoreactive neurons of layers I, II, and superficial III, were large fusiform, bipolar, or multipolar neurons possessing elongated dendritic arbor that spanned into adjacent cortical layers (Figs. 11A–J and 12A,B). Giant CR-immunoreactive multipolar neurons were observed in layer III of the temporal cortex (Fig. 12C). Fusiform and horizontally oriented neurons of the Cajal-Retzius type (Ramón y Cajal, 1911; Marin-Padilla, 1990) were observed in layer I of the frontopolar cortex (Fig. 12D,E). Occasionally, large bipolar and multipolar neurons were noted in layer VI in anterior and posterior lateral gyrus (Fig. 12F,G), representing the putative somatosensory and visual cortices, respectively.

**Golgi Preparations**

Golgi preparations of the three cortical regions studied (e.g. lateral gyrus, putative visual cortex; frontal magnocellular field, putative motor cortex; and ACC) revealed a large variety of spiny neuronal morphologies. However, it is important to note that absence in Golgi preparations of particularly neuron types does not mean these neurons were not present in the cortical region analyzed. Golgi staining results can vary considerably among regions with only a small percentage of neurons stained in each preparation. In the present study, we obtained a greater number of well-stained neurons in the cortex of the lateral gyrus and in the magnocellular cortex than in the ACC. As a consequence, our Golgi samples did not allow for a comprehensive determination of all neuronal morphologies belonging to a specific cortical area, but represent instead a qualitative description of a sample of neurons and the characteristics of their dendritic arbor from each analyzed block.

Typical pyramidal neurons of small and medium size with triangular or round somata were observed in all cortical layers in the putative visual and motor cortices, and in the ACC. These neurons possessed prominent apical dendrites, often bifurcating at some distance from the soma as they ascend toward the pial surface, thus providing two distinct apical shafts. The basilar skirt was relatively simple radiating in many directions for a short distance from the soma (Figs. 13Aa,Ac,Ba,F,G and 14A,D,E).

Magnopyramidal neurons were observed in layer V of the magnocellular frontal field, the putative motor cortex. They projected very long ascending apical dendrites that often bifurcated, at varying distances from the mostly triangular soma, into two branches (Fig. 15A). The basilar skirt spread in multiple directions (Fig. 14A,D).

Extraverted pyramidal neurons exhibiting the typical predominant subpial dendritic arborization (Sanides and Sanides, 1972) were observed in both superficial and deep layers (II, III, and V) of the putative visual and motor cortices. These neurons of small, and sometimes medium soma size, were characterized by a relatively large apical motor branch system compared with the basilar dendritic arbor. Interestingly, this neuronal type was also observed in deeper cortical layers (Fig. 13Ab,H and 14Cb,G,H).

Multiapical pyramidal neurons were mainly located in layer V and observed in both the putative visual and motor cortices. These neurons were characterized by a triangular or spherical soma, from which prominent and symmetric apical dendrites extended toward the pial surface, and by a simple, and often diminutive, basilar skirt with dendrites descending towards the white matter or projecting radially for a short distance from the soma (Figs. 13Bc,E and 14Ca,F).

Von Economo neurons (VENs) exhibiting the typical morphological features described in other species (Nimchinsky et al., 1995, 1999; Butti et al., 2009; Hakeem et al., 2009; Allman et al., 2010; Butti and Hof, 2010) were observed in layer V of the putative visual cortex (with a few in deep layer III; Fig. 13Bb). However, it is worth noting that VENs were observed in all cortical regions of the pygmy hippopotamus in Nissl-stained sections (see von Economo Neurons below, and Fig. 16 for further details on VEN morphologies and distribution).

Fork neurons, previously described in the cortex of humans and great apes (Ngowyang, 1932; Allman et al., 2010; Seeley et al., 2012), as well as elephants (Jacobs et al., 2011) and cetaceans (Hof and Van der Gucht, 2007), were observed in layer V of the putative visual cortex of the pygmy hippopotamus and were characterized by a thick soma that split into two thick ascending apical dendrites and by the presence of thin basal dendrites running laterally (Fig. 13D).

Large pyramidal neurons with a descending taproot were observed in layer V of the visual cortex. They presented a large fusiform soma from which a thick apical dendrite originated. Their basilar skirt was characterized by a central thick dendrite (the taproot; Jacobs et al., 2011) descending for a long distance towards the white matter and from which a dense array of small lateral dendrites extended radially (Fig. 13C).

**Von Economo Neurons**

VENs were observed in layer V of all cortical regions of the cerebral cortex of *H. liberiensis* including the typical regions of distribution of VENs (ACC, anterior insula, and frontopolar cortex), but also parietal, temporal, and occipital cortices. Even though VENs were abundant throughout all cortical areas, they were still restricted to layer V and, occasionally deep layer III, as seen in the species in which VENs have been described to date (Nimchinsky et al., 1995, 1999; Hof and Van der Gucht, 2007; Fajardo et al., 2008; Butti et al., 2009; Hakeem et al., 2009; Allman et al., 2010; Butti and Hof, 2010;
VENs in the pygmy hippopotamus showed the typical set of morphological features described in hominids, macaque monkeys, cetaceans, and elephants (von Economo, 1926; Nimchinsky et al., 1995, 1999; Butti et al., 2009; Hakeem et al., 2009; Allman et al., 2010; Evrard et al., 2012) (Fig. 16A–F). The morphology of VENs observed in the cortex of *H. liberiensis* resembled closely those that we described in

Fig. 11. Photomicrographs showing the distribution of CR-immunoreactive neurons in several cortical regions of the pygmy hippopotamus. Frontopolar cortex (A), cortex of the sigmoid gyrus (putative motor cortex) (B), cortex of the anterior lateral gyrus (putative somatosensory cortex) (C), suprasylvian cortex (putative auditory cortex) (D), anterior insular cortex (E), perisylvian cortex (F), posterior insular cortex (G), temporal cortex (H), anterior retrosplenial cortex (I), cortex of the posterior lateral gyrus (putative visual cortex) (J). Note the distribution of the CR-immunoreactive neurons mostly in the superficial layers I, II, and III. Scale bar = 400 µm.
Fig. 12. Photomicrographs showing the typical bipolar and fusiform morphology as well as large atypical morphologies of CR-immunoreactive neurons in the cortex of the pygmy hippopotamus. Bipolar and fusiform neurons in layer III of the posterior lateral gyrus (putative visual cortex; A); multipolar neuron in layer II of the frontopolar cortex (B); giant multipolar neuron in layer III of the temporal cortex (C); Cajal-Retzius neurons in layer I of the frontopolar cortex (D, E); large fusiform neuron in layer VI of the cortex of the posterior lateral gyrus (putative visual cortex; F); multipolar neuron in layer VI of the cortex of the anterior lateral gyrus (putative somatosensory cortex; G). Coronal sections in rostro-caudal order showing the location of panels 11A–11J, and 12A–12G (12H–12M). Scale bar = 100 μm.
several cetacean species (Hof and Van der Gucht, 2007; Butti et al., 2009). The most frequently observed morphology was characterized by a stout cell body with apical and basal dendrites almost as thick as the cell body (Fig. 16A,C,D,F). We also observed VENs with extremely slender cell bodies (Fig. 16B), resembling the morphologies mostly described in hominids (Nimchinsky et al., 1995, 1999; Allman et al., 2010), and fork neurons characterized by a soma divided into two thick apical dendrites and by a single and thick basal dendrite (Fig. 16E). Occasionally, VENs possessed a basal dendrite divided in two branches and a hint of a basilar skirt (Fig. 15B,C), as previously described in humans (Nimchinsky et al., 1995).

Fig. 13. Photomicrographs of Golgi-impregnated neurons observed in the lateral gyrus of the pygmy hippopotamus. (A) a, pyramidal neuron; b, extraverted pyramidal neuron; c, pyramidal neuron; (B) a, pyramidal neuron; b, von Economo neuron; c, multiapical pyramidal neuron; (C) large deep pyramidal neuron with a descending taproot; (D) fork neuron; (E) multiapical pyramidal neuron; (F-G) pyramidal neurons; (Ga) higher magnification insert of basilar dendrite showing spine density; (H) extraverted pyramidal neuron. Scale bar = 100 μm (A–H), and 50 μm (Ga).
DISCUSSION

The present study is the first to document the cortical organization of the brain of a hippopotamid, the pygmy hippopotamus, *H. liberiensis*. It is known that there are cytoarchitectural and neurochemical similarities between cetaceans and artiodactyls (Hof et al., 1999), and many recent phylogenetic analyses have supported a sister-group relationship between cetaceans and hippopotamids.

Fig. 14. Photomicrographs of Golgi-impregnated neurons observed in the frontal magnocellular cortex (A–D, F–H) and in the ACC (E) of the pygmy hippopotamus. (A, D) magnopyramidal neuron; (Aa) higher magnification inset of apical dendrite showing spine density. (B, E) pyramidal neurons; (C) a, multiapical pyramidal neuron; b, extraverted pyramidal neuron; (F) multiapical pyramidal neuron; (G, H) extraverted pyramidal neurons. Note the presence of bifurcating apical dendrites. Scale bars — 80 μm (A–C, F–H), 200 μm (D, E), and 50 μm (Aa).
In comparing brain structure between the pygmy hippopotamus and cetaceans, some features are primitive (pleisiomorphic) and shared widely with other cetartiodactyls and other mammals. Other features are specialized (apomorphic, derived) relative to other cetartiodactyls. For these, a feature present in only one group, either hippopotamus or cetacean, might reflect the amphibious or wholly aquatic lifestyle in that group, whereas a derived feature present in both groups could be a shared amphibious-aquatic specialization. Alternatively, such a feature might have evolved convergently in the two groups. Some judgment is needed in interpreting a feature as being an adaptation to an amphibious-aquatic habitat, because fossil evidence does not indicate whether the most recent common ancestor of hippopotamids and cetaceans was terrestrial or amphibious-aquatic.

**GI and CCA/BM Ratio**

For its size and degree of convolution, the external anatomy of the pygmy hippopotamus brain resembles that of other terrestrial artiodactyls, diverging extremely from the large size and high degree of convolution of the brain of small and large cetaceans (Kojima, 1951; Breathnach, 1955; Jacobs et al., 1971, 1979; Morgane et al., 1980; Marino et al., 2001a,c, 2002, 2003a,b, 2004a,b; Hof et al., 2005; Hof and Van der Gucht, 2007; Butti and Hof, 2010). This is confirmed by the calculation of the GI of the pygmy hippopotamus (1.84) that fits within the range of GI previously calculated for artiodactyls (Manger et al., 2012). Such value is considerably lower than the GI calculated by Manger et al. (2012) in cetaceans (average GI of six species = 5.43) and in pinnipeds (average GI of two species = 2.45). Comparison of the pygmy hippopotamus GI value to the log-transformed data indicating the relationship between brain mass and GI calculated by Manger et al. (2012), indicates that the brain of the pygmy hippopotamus appears to have the value of GI expected for an artiodactyl brain of its size. In their extensive analysis of GI, Manger et al. (2012) have found that the three groups of marine mammals that they have studied (cetaceans, pinnipeds, and manatees) possess GI values that differ consistently from those of terrestrial mammals. In this respect, the pygmy hippopotamus fits well into the group of artiodactyls and differs from cetaceans (Table 4).

Quantitatively, the values of the CCA/BM ratio obtained with both the method of Tarpley and Ridgway (2000) (CCA/BM = 0.737; Table 2) and the method of Manger et al. (2010) (CCA/BM = 0.22; Table 3) showed that the corpus callosum was larger in comparison to the mass of the hemispheres than in cetaceans. The
latter are, in fact, characterized by a relatively small and thin corpus callosum (with CCA/BM ratios ranging from 0.079 in the killer whale, *Orcinus orca*, to 0.431 in the Ganges river dolphin, *Platanista gangetica* as in Tarpley and Ridgway, 2000, and from 0.11 in the Northern right whale dolphin, *Lissodelphis borealis* to 0.15 in the bottlenose dolphin, *Tursiops truncatus*, and other small odontocetes as in Manger et al., 2010; Tables 2 and 3). The same conclusion can be drawn when comparing the CCA/BM value of the pygmy hippopotamus, obtained as in Manger et al. (2010), with the values reported by these authors for a group of artiodactyls (CCA/BM ratios spanning from 0.16 in the dromedary, *Camelus dromedarius*, to 0.18 in the reindeer, *Rangifer tarandus*, including a value of 0.17 in the river hippopotamus, *H. amphibius*) or other eutherian mammals of comparable brain size (see Manger et al., 2010; Table 3). As such, the CCA/BM ratio for the pygmy hippopotamus fell above the range found by Manger and colleagues (2010) for cetaceans (0.11–0.15), for other aquatic species including pinnipeds and sirenians (0.13–0.17), and for artiodactyls (0.16–0.18), including artiodactyls of comparable brain mass (e.g., reindeer, *R. tarandus*, and scimitar-horned Oryx, *Oryx dammah*). The pygmy

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**Fig. 16.** Photomicrographs showing different morphologies of von Economo neurons (VENs) in Nissl-stained sections through all cortical regions of the pygmy hippopotamus. Note the presence of VENs with stout cell bodies (A, C, D, F) similar to what has been described in cetaceans (Butti et al., 2009) and the more occasional presence of very elongated and slender VENs (B), comparable to those seen in hominids. Note also the presence of fork neurons (A, E). VENs are indicated by arrows and fork neurons by arrowheads. Scale bar = 50 μm.
hippopotamus has the highest CCA/BM ratio among artiodactyls examined to date and such value was comparable only to the value obtained in certain perissodactyls (Rhinoceros, Diceros bicornis), carnivores (brown bear, Ursus arctos), numerous primates, and two females Indian elephants, Elephas maximus (Manger et al., 2010). Our results, together with those reported by Manger et al. (2010) and Tarpley and Ridgway (2000), indicate that the brain of the pygmy hippopotamus is very different, in terms of mid-sagittal cross-sectional area of the corpus callosum, from that of cetaceans. It thus appears that cetaceans, as previously suggested, may have evolved a different morphology from other mammals (Manger et al. 2010). Interestingly, the CCA/BM value found in our specimen using the method of Tarpley and Ridgway (2000) is comparable to the values reported by these authors for the Florida manatee, Trichechus manatus manatus (CCA/BM = 0.829). However, the CCA/BM ratio reported by Manger et al. (2010) for the Amazonian manatee, Trichechus inunguis (CCA/BM = 0.17), the smallest species of manatees, is lower than the value calculated with the same method for the pygmy hippopotamus (0.22). These contrasting results could be due to a specific feature of this particular species of manatees or, more likely, to lack of methodological precision, as most of these studies are based on a single specimen due to their rarity. As such, differences found between the pygmy hippopotamus and other eutherian mammals of comparable brain size, and aquatic mammals in general, deserve additional research. This could be done ideally by increasing the sample size of a given species and by comparing the pygmy hippopotamus to the larger river hippopotamus, H. amphibius, to determine if the CCA/BM results are due to an outlier specimen or whether they represent a general pattern displayed by the pygmy hippopotamus or hippopotamuses in general.

Volumetric Measurements, CQ, and CI

In the pygmy hippopotamus the cerebellum was not as large relative to the cerebral hemisphere as in cetaceans (occupying 9.72% of the total brain volume in the pygmy hippopotamus, a range of 13.77% to 14.98% in the white-sided dolphin, Lagenorhynchus acutus, 15.07% in the common dolphin, Delphinus delphis, and 15.05% in the bottlenose dolphin, T. truncatus; Fig. 2, Table 1; Breathnach, 1980; Marino et al., 2000). This conclusion is reflected in the calculation of the cerebellar quotient (CQ) of the pygmy hippopotamus (CQ = 0.80; Table 4) based on the equation given by Maseko et al. (2012). The cerebellar volume of the pygmy hippopotamus was 0.8 times the size that would be expected based on the mammalian baseline calculated by Maseko et al.; a direct comparison with a cetacean of similar brain size, the franciscana (Pontoporia blainvillei; Maseko et al., 2012) shows that the relative amount of neural tissue dedicated to the cerebellum is much reduced in the pygmy hippopotamus compared with the cetacean. Calculation of the cerebellar index (cerebellar volume/brain volume) for the two species (pygmy hippopotamus CI = 0.97; Pontoporia CI = 1.52) supports this conclusion. The CQ of the pygmy hippopotamus is lower than that of each species of cetaceans examined to date (Maseko et al., 2012) and it is consistent with an enlarged cerebellum being, within cetartiodactyls, a characteristic that evolved after the transition from a terrestrial to a fully aquatic environment. Our calculation of CQ and CI in additional species of artiodactyls and carnivores (both aquatic and terrestrial; Table 4) shows that these values for the pygmy hippopotamus fit well into the range of CQs and CIs of artiodactyls and that they are also comparable to CQs and CIs of carnivores of comparable brain mass (harbor seal, Phoca vitulina, and African lion, Panthera leo; Table 4).

The hippocampus was larger, in both absolute size and size relative to the whole brain volume, in comparison to what has been reported in cetaceans (e.g., white-sided dolphin: left hippocampus volume range = 0.544 to 1.043 cm³; right hippocampus volume range = 0.462 to 0.967 cm³; Table 1; Breathnach and Goldby, 1954; Breathnach, 1960; Filimonoff, 1965; Gruenberger, 1970; Jacobs et al., 1979; Morgane et al., 1982; Schwerdtfeger et al., 1984; Marino et al., 2003b, 2004a,b; Hof and Van der Gucht, 2007; Montie et al., 2008). In addition, the entorhinal cortex in the pygmy hippopotamus did not appear as enlarged as in cetaceans. When comparing the volume of the hippocampus measured in the present study to that of the few artiodactyls for which data are available (range 2,667.84–8,577.12 mm³; Reep et al., 2007), it becomes apparent that hippocampal volume in the pygmy hippopotamus (8,530 cm³) is among the highest values reported for artiodactyls. More hippopotamus specimens are necessary to confirm such a conclusion.

All cetacean species reported to date have exhibited a relatively small hippocampus, which putatively indicates a possible adaptation to the aquatic environment. This unique feature of the cetacean brain has been suggested to reflect a different organization of the circuits supporting memory processing, compared with terrestrial mammals (Squire and Zola-Morgan, 1981; Zola-Morgan, 1983; Hof and Van der Gucht, 2007). For other aquatic mammals, the sirenians, discordant results have been reported on the size of the hippocampus. Some authors have reported a reduced hippocampus in sirenians as in cetaceans (Oelschläger and Oelschläger, 2002; Marino, 2007), whereas others have noted the hippocampal volume in sirenians to be comparable to that of many terrestrial mammals (Reep et al. 2007). Larger sample sizes and comparison with the structure of the hippocampal formation of amphibia carnivores (pinnipeds: seals, walruses, and sea lions; and otters) could help elucidate this matter further.

Differences in the development of the hippocampal formation between cetaceans and their closest relative, the pygmy hippopotamus (as well as other artiodactyls), could be related specifically to differences in spatial memory and aquatic navigational demands. The anatomical structure of the hippocampal formation in aquatic mammals might reflect migratory patterns insofar as migration is considered a major determinant of the size of the hippocampus in birds (Pravosudov et al., 2006). To investigate this matter further, marine mammals clearly represent an excellent group of study because of the range of variation in their migratory behavior (for review see Stern, 2009). Our observation of an enlarged hippocampus in the pygmy hippopotamus, even compared with other artiodactyls (Table 1), is consistent with a diminutive hippopotamus being an adaptation to the obligatory aquatic lifestyle of cetaceans.
When considering the volumetrics of the cerebellum and hippocampus the brain of *H. liberiensis* can be considered an artiodactyl-like brain. However, a different situation arises when considering the corpus callosum, as the CCA/BM ratio is higher than any artiodactyl (and cetacean) analyzed to date.

**Cytoarchitecture and Chemoarchitecture**

The pygmy hippopotamus cortex lacks an internal granular layer (layer IV: Figs. 3–10) as cetaceans (Morgan et al., 1988; Glezer and Morgane, 1990; Hof et al., 2005; Hof and Van der Gucht, 2007; Oelschläger and Oelschläger, 2008). There has been a long debate about the presence or absence of an internal granular layer (layer IV) in artiodactyls and cetaceans and, to date, an incipient layer IV has been described in the primary visual cortex of certain artiodactyls (Rose, 1942), but it is generally recognized that the cetacean neocortex lacks this layer. In this respect, the cortex of the pygmy hippopotamus is cetacean-like and very different from the cortex of other artiodactyls. The absence of a layer IV may indicate a specific wiring pattern (Hof and Van der Gucht, 2007), possibly associated with a shift of thalamic inputs to layer I rather than deeper layers (Ferrer and Perera, 1988; Glezer and Morgane, 1990). Second, the cerebral cortex of the pygmy hippopotamus contains extensive clustering of neurons in layer II, not only in the anterior insular cortex, but also in large portions of the occipital cortex (Fig. 10). Although neuronal clustering of layer II is observed in the anterior insula of both odontocetes and mysticetes (Jacobs et al., 1984; Manger et al., 1998; Hof and Van der Gucht, 2007; Butti and Hof, 2010), this finding is important because, to date, clustering of layer II in the occipital cortex has only been formally documented in two balaenopterids, namely the humpback whale and the fin whale (*Balaenoptera physalus*) (Hof and Van der Gucht, 2007). Such a pattern was suggested to be a rostral specialization and to represent a specific type of cortical connectivity in the occipital cortex, possibly shaped by thalamic afferents in the absence of layer IV (Hof and Van der Gucht, 2007). Our results suggest that, although the clustering pattern in layer II of the anterior insula has been retained by all cetaceans during the adaptation to the aquatic life, the modularity of layer II in the occipital cortex, and its potentially related cortical connectivity, may have been retained in certain species of cetaceans (rorquals) but not in others (odontocetes). Alternatively this modular architecture of the occipital cortex might have evolved independently in the pygmy hippopotamus and rorquals. Observations in a limited number of species support the presence of clustering of layer II in the occipital cortex of other artiodactyls (Hof, personal communication). However, the degree to which such clustering occurs and the distribution of this feature among cetartiodactyls has not yet been formally documented. This issue requires an in depth study which is beyond the aim of this article.

We also noted that the cortex of the pygmy hippopotamus showed layer II clustering beyond the anterior insular and occipital cortices. The putative somatosensory cortex, in this species, is located consistently with what has been defined as somatosensory cortex in cetaceans (Lende and Welker, 1972; Hof and Van der Gucht, 2007) and is characterized by a high degree of layer II clustering throughout its entire extent. This trait can be easily used to define its cytoarchitectural boundaries from adjacent cortical fields (see above, Frontopolar Cortex and Frontal Convexity and Parietal Cortex sections for details). Such an extensive clustering could be a cortical specialization of hippopotamids, partially retained during the adaptation to the aquatic environment by some cetacean groups. In this context, the analysis of the presence and distribution of layer II islands in the cortex of the larger river hippopotamus, and other artiodactyls, in comparison to *H. liberiensis*, should elucidate the possible function and evolutionary significance of such pattern.

In terms of distribution of neocortical fields on the surface of the hemisphere, the brain of the pygmy hippopotamus is probably comparable to that of cetaceans and artiodactyls, but the distribution of the visual and somatosensory cortices deserves some additional comments. In fact, although the putative motor and somatosensory cortices are arranged consistently with their localization in cetaceans (Lende, 1968; Lende and Welker, 1972), the putative somatosensory cortex of the pygmy hippopotamus extends caudally for a greater length of the lateral gyrus, confining the putative visual cortex to only its most caudal part, as seen in artiodactyls, and in contrast to cetaceans where the somatosensory cortex is limited to a rather small area in the frontal convexity of the hemisphere.

The putative motor cortex of the pygmy hippopotamus was very similar to that recognized both electrophysiologically and cytoarchitecturally as the motor cortex in cetaceans (Kojima, 1951; Lende, 1968; Kesarev and Malofeeva, 1969; Ladygina and Supin, 1977; Hof and Van der Gucht, 2007). It is interesting to note that in cetaceans, the belt of motor cortex in the frontal convexity extends rostro-caudally and is separated laterally from the somatosensory cortex by the cruciate sulcus (Kesarev and Malofeeva, 1969; Lende and Welker, 1972; Sokolov et al., 1972; Ladygina and Supin, 1977; Glezer, 2002). However, although the cruciate sulcus develops in a rostrocaudal direction in cetaceans, possibly as a consequence of the rostroventral rotation that the cetacean brain undergoes during development, the cruciate sulcus develops laterally in the pygmy hippopotamus, reaching the most dorsal aspect of the anterior Sylvian cortex.

While in cetaceans almost the totality of the lateral gyrus is occupied by visual cortex (Sokolov et al., 1972; Ladygina and Supin, 1977; Revischin and Carey, 1989; Hof and Van der Gucht, 2007), in the pygmy hippopotamus the putative visual cortex occupied only the most posterior aspect of the lateral gyrus. This location is reminiscent of the location of the visual cortex in many terrestrial mammals such as carnivores (Sanides and Hoffmann, 1969; Sherk, 1986; Payne, 1993; Innocenti et al., 2002; Manger et al., 2004) and other artiodactyls (Campbell, 1965; Alouf, 1929; Rose, 1942).

The distribution of CR-immunoreactive neurons in the cortex of the pygmy hippopotamus is relatively uniform throughout cortical regions and comparable to what has been observed in the cortex of cetaceans and several species of artiodactyls (Glezer et al., 1993, 1998; Hof et al., 1996a, b, 1999; Fig. 11). In addition, the morphologies of CR-immunoreactive neurons in the pygmy hippopotamus are similar to what has been observed in cetaceans (Fig. 12).
However, the frequent presence of giant CR-immunoreactive multipolar neurons in deep layers is uniquely shared with artiodactyls, to the exclusion of cetaceans. Additionally, the CR-immunoreactive multipolar neurons in layer III of the temporal cortex (Fig. 12C) resembled those observed in layer III and V of the primary motor cortex of carnivores (for review see Hof et al., 1999). The large CR-bipolar and multipolar neurons of layer VI (Fig. 12F,G) of the putative somatosensory and visual cortices, were reminiscent of the large multipolar neurons described in layer VI of the giraffe (Giraffa camelopardalis), llama (Lama glama), and Bactrian camel (Camelus dromedarius) (Hof et al., 2000). Such pattern of distribution and morphologies of CR-immunoreactive neurons is interesting in light of the recent morphological and molecular evidence of phylogenetic relatedness between hippos—the closest terrestrial relatives. Moreover, such an evolutionary transition between a “general” pattern of distribution and morphologies shared with artiodactyls (at least at a laminar level and in the species observed so far).

Sparse and rare VENs have also been observed across the cortical mantle in another aquatic mammal, the Florida manatee (Trichechus manatus manatus) (Butti and Hof, 2010), which is closely related to elephants (Kellogg et al., 2007). Such differences are consistent with a reorganization of the distribution of VENs into a regionally specific pattern. This distribution, which might correlate with the presence of shared evolutionary traits between cetaceans and artiodactyls (Glezer et al., 1992, 1993, 1998; Hof et al., 1996a, 1999, 2000). In fact, the CR immunoreactivity in the pygmy hippopotamus cortex makes it impossible to compare the distribution of VENs between the pygmy hippopotamus and these species. VENs are present also in layer II of the frontopolar cortex of certain artiodactyls described so far. Such observation is consistent with a laminar distribution of VENs in the pygmy hippopotamus comparable to what described in hominids, elephants, and cetaceans, but different from artiodactyls (at least at a laminar level and in the species observed so far).

Another intriguing observation is the presence, in pygmy hippopotamus cortex, of a neuronal morphology that is reminiscent of the taproot neuron (called the “matriarch neuron” by Jacobs et al., 2011; Fig. 13) observed in the frontal cortex of the African elephant (Jacobs et al., 2011). These neurons, possessing a complex and extensive dendritic system may represent extreme adaptations of pyramidal neurons, much the same as other large cortical neurons in primates (e.g., Betz, Meynert, and von Economo's spindle cells) (Jacobs et al., 2011, p 292). Based on their peculiar morphology, Jacobs et al. (2011, p 292) suggested that these neurons might sample a wide range of cortical inputs and exert modulatory field effect in the surrounding neuropil, potentially contributing to associative cognitive processes.

In addition, the presence of extrverted neurons in layers II, III, and V in the cortex of the pygmy hippos is worth noting. Such neurons with apical dendrites spreading into layer I have been reported in the cortex of cetaceans, but exclusively in...
layer II (Morgane et al., 1985; Ferrer and Perera, 1988; Morgane et al., 1988), and are generally considered a conservative feature of the cetacean neocortex (Morgane et al., 1985, 1988; Glezer, 1988) as a comparable neuronal morphology was previously observed in mammals such as bats, hedgehogs, opossums, xenarthrans and afrotherians (Sanides and Sanides, 1972; Sherwood et al., 2009). In cetaceans extraverted neurons have been proposed by Glezer and Morgane (1990) to represent strong neuronal inputs into layer I of the axodendritic type which, thus, resemble the pattern of input seen in allocortical formations (Glezer and Morgane, 1990; p 418).

Our results show that such neuronal morphology is present in hippopotamids as well (Fig. 13Ab,H; Fig. 14Ch,G,H). As such, this morphology appears to have developed in cetartiodactyla before their transition to an aquatic environment. However, the distribution of extraverted pyramidal neurons is different in the hippopotamus and cetaceans, being restricted to layer II in the latter, but similar to xenarthrans and afrotherians (Sherwood et al., 2009). The functional significance of the different distribution of this neuronal type is difficult to assess and the analysis of neuronal morphologies in the river hippopotamus, as well as other terrestrial artiodactyls, could elucidate this matter further.

Methodological Limitations and Future Directions

Methodological limitations regarding the Golgi staining of postfixed tissue have been discussed elsewhere (Jacobs et al., 2011). The major limitation in the present study was the impossibility to trace completely dendritic systems and, thus, to provide a quantitative description of the neuronal morphologies identified. For this reason our results provide an initial qualitative description of the neuronal morphologies identified. This morphology appears to have developed in cetartiodactyla before their transition to an aquatic environment. However, the distribution of extraverted pyramidal neurons is different in the hippopotamus and cetaceans, being restricted to layer II in the latter, but similar to xenarthrans and afrotherians (Sherwood et al., 2009). The functional significance of the different distribution of this neuronal type is difficult to assess and the analysis of neuronal morphologies in the river hippopotamus, as well as other terrestrial artiodactyls, could elucidate this matter further.

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