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Neuron numbers in sensory cortices of five delphinids compared to a physeterid, the pygmy sperm whale

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Abstract

With its large mass and enormous gyrification, the neocortex of whales and dolphins has always been a challenge to neurobiologists. Here we analyse the relationship between neuron number per cortical unit in three different sensory areas and brain mass in six different toothed whale species, five delphinids and one physeterid. Cortex samples, including primary cortical areas of the auditory, visual, and somatosensory systems were taken from both hemispheres of brains fixed in 10% buffered formalin. The samples were embedded in paraffin, sectioned at 25 μm thickness and stained with cresyl violet. Because cortical thickness varies among toothed whale species, cell counts were done in cortical units measuring 150 μm in width, 25 μm in thickness, and extending from the pial surface to the white matter. By arranging the delphinid brains according to their total mass, 834–6052 g, we found decreasing neuron numbers in the investigated areas with increasing brain mass. The pigmy sperm whale (*Kogia breviceps*), a physeterid with an adult brain weight of 1000 g had a distinctly lower neuron number per cortical unit. As had been expected, an increase in adult brain weight in delphinid cetaceans (family Delphinidae) is not correlated with an increase in neuron number per cortical unit.

Keywords: Neuron number; Cortical unit; Auditory, visual, somatosensory cortex; Delphinid, physeterid, Cetacea

1. Introduction

Whales and dolphins (Cetaceans) are marine mammals which descended from ancient carnivorous land-living hoofed animals more than 50 million years ago [3]. These ancestors adapted gradually to their new environment, e.g. by establishing a spindle-shaped body profile, reducing their pelvic girdle and hindlimbs [2,4]. Among the toothed whales (Odontoceti) one family, the Delphinidae or dolphins, evolved a new and highly sophisticated sonar system for underwater orientation and navigation. Among the most fascinating characteristics of delphinids are their large brains, both in absolute and relative terms, and their extremely convoluted neocortex [10,12,15]. In principle, dolphin brains show the typical mammalian organization and seem to be as complicated morphologically as those of other mammals of the same size. The smaller delphinid species may have an adult body mass in the 50–100 kg range, the maximal size is attained in killer whales (*Orcinus orca*) where adult body mass may reach 5000 kg or more.

In another toothed whale family, the Physeteridae, which comprise the (giant) sperm whale (*Physeter macrocephalus*), adult males may attain a brain mass of up to 9200 g [8]. Giant cetaceans, however, are difficult to interpret because their brains, although approximating 10 kg in total mass, is dwarfed by the huge body (35 t). We did not have a giant sperm whale brain suitable for study but could use a small member of the family Physeteridae, the pygmy sperm whale (*Kogia breviceps*) which is in the delphinid size range.

In this paper, we compare the pygmy sperm whale with five different species of delphinids. We focus on the correlation between neuron number per neocortex unit (i.e. the number of perikarya below a defined area of the cortex surface)

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and the brain mass in five adult individuals of five species of delphinids with a body size ranging from 90 to 3273 kg.

2. Material and methods

The brains of six adult specimens belonging to six species of toothed whales (Odontoceti), including the common dolphin (*Delphinus delphis*), pigmy sperm whale (*K. breviceps*), bottlenose dolphin (*Tursiops truncatus*), long-finned pilot whale (*Globicephala macrorhynchus*), false killer whale (*Pseudorca crassidens*) and killer whale (*O. orca*) were investigated. The brains came from strandings and oceanaria where the animals died of natural causes but were accessible for prompt fixation. They were fixed in 4% formaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 (10% buffered formalin). Samples were taken from the primary auditory, the somatosensory and visual cortices of both hemispheres following electrophysiological mapping done in the 1970s [10,13]. They were embedded in paraffin, cut at 25 μm, mounted on microslides, deparaffinized and stained for cresyl violet (Nissl) before cover-slipping. Cell counts were done with an Olympus BHS microscope equipped with a dry 20× objective using a 10× eyepiece. In each sample, four cortical units from two different histological sections were analysed, each cortical unit measuring 150 μm in width, 25 μm in thickness and extending from the pial surface to the grey/white transition. In order to obtain reliable results, the correct position of the frame in the middle of a gyrus was always ascertained. Placements of the frame either too close to the crown or to the bottom of a gyrus resulted in over- and underestimations of neuron numbers, respectively. This is due to a distorted laminar pattern: at the crown of a gyrus the inner cortical layers are compressed while the outer layers are expanded and the inverse situation is given at the bottom of a fissure.

We only accepted neurons which had their nucleolus within the framework of the cortical unit in question. In order to confirm that only neurons were counted, we double-labelled adjacent sections for gliocytes using immunohistochemical techniques and cresyl violet counterstain. These sections were incubated in 0.3% H_2O_2 for 30 min, rinsed with phosphate-buffered saline (PBS) and incubated for 12 h at room temperature in primary polyclonal antibody against GFAP (Glial Fibrillary Acidic Protein, Sigma) obtained from rabbit at a dilution of 1:1000. The following procedures involved incubation for 90 min in the secondary biotinylated goat-anti-rabbit antibody, incubation for 2 h in avidin–biotinylated enzyme complex (ABC kit®, Vector Laboratories) and incubation in 0.05% diaminobenzidine solution for 3 min followed by the addition of 3% H_2O_2 for 30 s. After stopping the chromogen reaction in phosphate buffer (PB), the sections were dried overnight. Finally, they were counterstained for cresyl violet and coverslipped. All cell counts were carried out by two independent groups of collaborators. The results were compared later and the differences checked for reliability.

As to the neuron counts, analysis of the sections double-stained for GFAP and cresyl violet revealed good correspondence with the routine sections, as a confirmation that only neurons were counted. Correlations were analysed with Pearson's correlation coefficient (SPSS, version 11.0).

3. Results

From each cortical sample we analysed a total of four details from different sections and then calculated the arithmetic mean for every hemisphere. The dispersion of total neuron numbers showed a medium deviation of 13.8% for *D. delphis* (*Dd*) (range: 1–44%), 15.8% for *K. breviceps* (*Kb*) (range: 1–43%), 8.9% for *T. truncatus* (*Tt*) (range: 2–28%), 10% for both *G. macrohynchus* (*Gm*) and *P. crassidens* (*Pc*) (range: 2–24%). The medium variation within *O. orca* (*Oo*) was the lowest with 6.6% (range: 1–15%).

The neuron counts of the two groups of collaborators were within the normal standard deviation and therefore trustworthy. These results (outlined in Table 1 and depicted in Fig. 1a–d) show that increasing brain mass is correlated with decreasing neuron number per standard cortical unit.

The only significant correlation between brain weight and mean neuron number was found for the somatosensory cortex (Fig. 1b: $p < 0.05$, $r = 0.27$). The other cortices and the overall statistics were not significant but showed a similar trend in terms of decreasing neuron density with increasing brain mass.

From Table 1 it is evident that the data of the physeterid *K. breviceps* constitute an important outlier in all analyses. If

Table 1
Average neuron numbers

Species	Sex	Body weight (kg)	Brain mass (g)	Factor	aud R	vis R	som R	aud L	vis L	som L	Average
D. delphis (*Dd*)	F	90	834	1	191	226.75	167.5	315	192	170.5	210.46
K. breviceps (*Kb*)	–	368	1000	1.2	150	60.5	95.5	104.5	127	107.5	107.5
T. truncatus (*Tt*)	F	173	1302	1.56	125.25	140	123.25	151.75	159.75	144.5	140.75
G. macrorhynchus (*Gm*)	M	–	2733	3.28	121.5	104.5	93.75	135.25	115.25	111.5	113.63
P. crassidens (*Pc*)	F	310	4307	5.16	113.25	89	76.75	95	70.75	100.25	90.83
O. orca (*Oo*)	M	3273	6052	7.26	71	79.25	42	63.5	88.25	48.5	65.42

aud: auditory; vis: visual; som: somatosensory cortex; L: left; R: right.

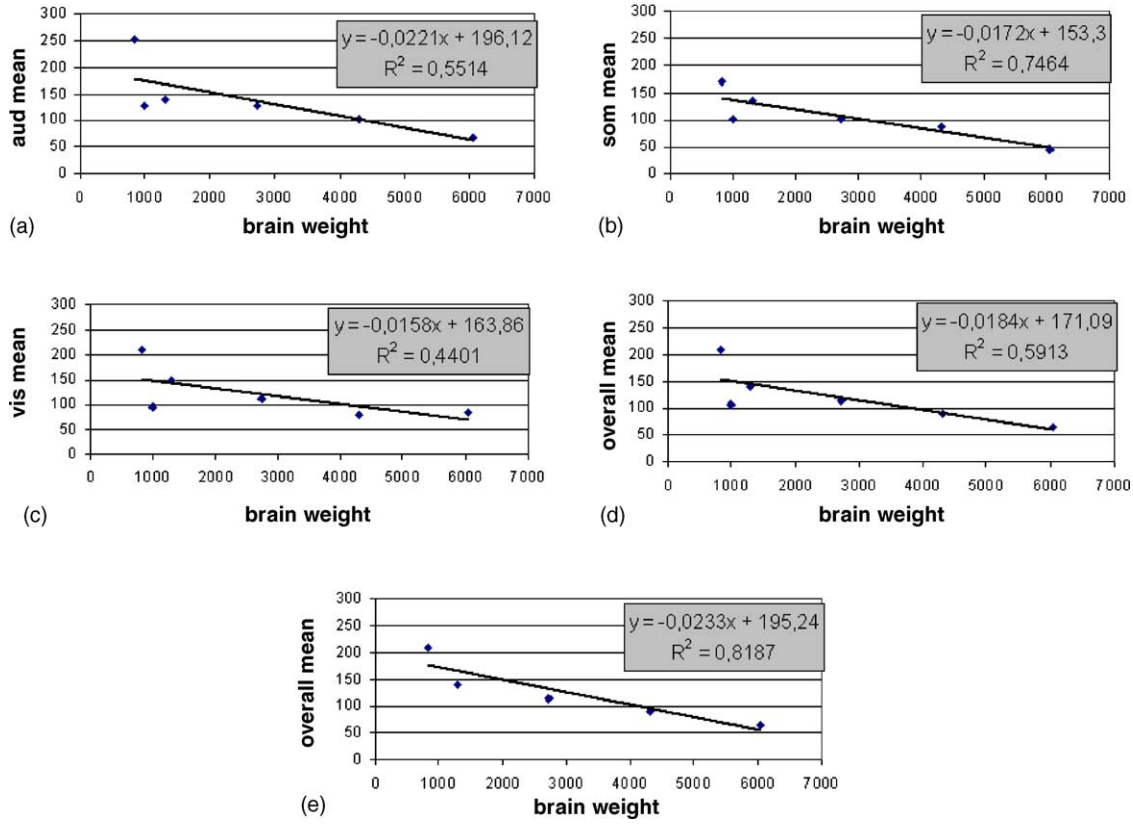

Fig. 1. (a) Brain weight vs. auditory neuron number; (b) brain weight vs. somatosensory neuron number; (c) brain weight vs. visual neuron number; (d) brain weight vs. overall neuron number; (e) brain weight vs. overall neuron number (*K. breviceps* omitted).

this data point is omitted, we obtain a significant regression for the remaining odontocetes (Fig. 1e: $p = 0.035$, $r = 0.905$).

Analysis of Nissl-stained sections counterstained for glia (GFAP) confirmed that only neurons were counted since none of the immunolabelled cells showed characteristics of neurons.

4. Discussion

The most important result of the present study is that, in the family Delphinidae, increasing brain mass is inversely related to neuron number per cortical unit. This correlation was found in all three sensory cortical systems. The member of the family Physeteridae, the pygmy sperm whale (*K. breviceps*), showed a somewhat different organization with respect to neuron number per cortical unit that might be explained by its distinct phylogenetic relationship (see below). It is difficult to compare our data with older literature on this topic [5,17] because their measurements are based on neuron density (neurons/mm^3), whereas our data are based on a 3D framework with one dimension (depth of the cortical grey matter) changing with every sample and species. In contrast we used cortical units for our analysis which are independent from the cortical thickness.

In view of the fact, that delphinids hunt and communicate via ultrasound and sound, respectively, and also at night and in murky waters, it is widely held that the acoustic/auditory system should be the dominant source of information for these animals [1,13]. Thus, e.g., in the bottlenose dolphin, many components of the auditory system are enlarged [14,18] and the primary auditory field encompasses a substantial proportion of the total neocortical surface [9,10]. Nevertheless, the neuron number per standard cortical unit was not different for the three sensory systems. This implies that in this case an increase in functional significance is associated with the enlargement of the corresponding area, not with a higher neuron number per cortical unit. The obvious left/right asymmetry in the auditory cortex of the brain of *D. delphis* can be seen as an outlier due to its singularity.

The data from *K. breviceps* comprise outliers in all three sensory systems. This species belongs to the family Physeteridae (sperm whales), which also includes the dwarf sperm whale (*Kogia sima*) and the giant sperm whale (*P. macrocephalus*). Within Cetacea, the sperm whales represent an ancient evolutionary line; i.e. they go back to the middle of the Oligocene period about 30 million years ago [3] when the odontocetes divided into different groups. All other whales investigated here belong to the family Delphinidae (dolphins) and are closely related to the fossil Kentriodontidae appearing in the middle of the Miocene period about 15 million years ago. Extant physeterids are deep-divers and might show appropriate specialisations as, e.g., a higher percentage of protective glia and thus a lower percentage of neurons in their

brains in order to cope with the lack of oxygen for up to 60 min and more in the giant sperm whale [16].

Among the five delphinid species, neuron number per cortical unit is inversely related to total brain mass. Allometric analyses of mammalian brains had revealed earlier that cerebral cortex volume increases disproportionately with brain size but that the proportion of cortical gray matter to total cortical volume decreases in larger brains [7]. In other words, the ratio between white matter and total cortical volume (compared to other mammals) is very high in dolphins because of the considerable size of their brains whereas their ratio between grey matter and cortical volume is lower [6]. Together these data imply that large-brained mammals tend to have disproportionately low grey matter volumes and high percentages of white matter. The mechanism guiding these allometric phenomena is not exactly known yet. One possibility of explanation is the so-called 'gyral-window hypothesis' which emerged out of the model proposed by Prothero and Sundsten [11]. In short, this hypothesis holds that an increase in brain mass enlarges cortical surface, which then results in an increase in gyrification. A further increase in brain mass and/or cortical surface is only possible by deepening the cortical fissures. Since, however, the neurons within a gyrus are in need of axonal connectivity with other parts of the brain, gyral width cannot be compressed below a certain value. This value defines an absolute limit for the maximal size of mammalian brains which is at about 10 kg [11]. Indeed, today's giant sperm whales and killer whales exhibit brains of about this value. This gyral-window hypothesis could therefore support the phenomenon of smaller neuron numbers in cortical units of larger brains.

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