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Although modern baleen whales still possess a functional olfactory systems that includes olfactory bulbs, cranial nerve I and olfactory receptor genes, their olfactory capabilities have been reduced profoundly. This is probably in response to their fully aquatic lifestyle. The glomeruli that occur in the olfactory bulb can be divided into two non-overlapping domains, a dorsal domain and a ventral domain. Recent molecular studies revealed that all modern whales have lost olfactory receptor genes and marker genes that are specific to the dorsal domain, and that a modern baleen whale possess only 60 olfactory receptor genes. Here we show that olfactory bulb of bowhead whales (*Balaena mysticetus*, Mysticeti) lacks glomeruli on the dorsal side, consistent with the molecular data. In addition, we estimate that there are more than 4,000 glomeruli in the bowhead whale olfactory bulb. Olfactory sensory neurons that express the same olfactory receptor in mice generally project to two specific glomeruli in an olfactory bulb, meaning that ratio of the number of olfactory receptors : the number of glomeruli is approximately 1:2. However, we show here that this ratio is not applicable to whales, indicating the limitation of mice as model organisms for understanding the initial coding of odor information among mammals.

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Organization and distribution of glomeruli in the bowhead whale olfactory bulb

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Abstract

Although modern baleen whales still possess a functional olfactory systemsthat includes olfactory bulbs, cranial nerve I and olfactory receptor genes, their olfactory capabilities have been reduced profoundly. This is probably in response to their fully aquatic lifestyle. The glomeruli that occur in the olfactory bulb can be divided into two non-overlapping domains, a dorsal domain and a ventral domain. Recent molecular studies revealed that all modern whales have lost olfactory receptor genes and marker genes that are specific to the dorsal domain, and that a modern baleen whale possess only 60 olfactory receptor genes. Here we show that olfactory bulb of bowhead whales (*Balaena mysticetus*, Mysticeti) lacks glomeruli on the dorsal side, consistent with the molecular data. In addition, we estimate that there are more than 4,000 glomeruli in the bowhead whale olfactory bulb. Olfactory sensory neurons that express the same olfactory receptor in mice generally project to two specific glomeruli in an olfactory bulb, meaning that ratio of the number of olfactory receptors : the number of glomeruli is approximately 1:2. However, we show here that this ratio is not applicable to whales, indicating the limitation of mice as model organisms for understanding the initial coding of odor information among mammals.

Introduction

Terrestrial mammals generally possess a well-developed sense of smell that can discriminate millions of odors using hundreds or thousands of olfactory receptors (ORs) (Nei et al. 2008). Odorants are detected by ORs expressing in the olfactory sensory neurons (OSNs), and the OSNs are projected to the glomeruli of the olfactory bulbs (OBs). Each OSN express only one *OR* gene (Serizawa et al. 2004), and OSNs expressing the same OR converge their axons to a specific set of glomeruli in the olfactory bulb (OB) (Mombaerts et al. 1996). Using mice as model organisms, it is reported that any 1 OR is typically represented by two glomeruli (Ressler et al. 1994; Vassar et al. 1994). This indicates that the number of glomeruli in the OB is approximately twice that of the number of *OR* genes in its genome.

The glomerular layer of the OB can be classified into two domains, the dorsal (D) domain and the ventral (V) domain, based on the expression patterns of domain-specific marker genes (Imai & Sakano 2007). The D domain is defined by the expression of the *OMACS* gene (Imai & Sakano 2007; Oka et al. 2003), and the V domain is defined by the expression of the *OCAM* gene (Imai & Sakano 2007; Yoshihara et al. 1997). All mammalian *OR* genes can be classified into two subfamilies, class I and class II, based on sequence similarities (Niimura & Nei 2006). The OSNs expressing class I ORs are projected to the D domain of the OB, while OSNs expressing class II ORs are projected to both D and V domains (Imai & Sakano 2007; Tsuboi et al. 2006).

Cetaceans are an order of mammals that originated in the early Eocene epoch and that was derived from terrestrial artiodactyls (Thewissen et al. 2009). Extant cetaceans are classified into two monophyletic suborders, Odontoceti (toothed whales) and Mysticeti (baleen whales). Modern cetaceans are known to have reduced the olfactory capabilities profoundly during their evolution, and odontocetes have no nervous system structures that mediate olfaction (Oelschläger et al. 2010). On the other hand, mysticetes have a fully equipped olfactory system and OB (Thewissen et al. 2011), but the number of functional *OR* genes is remarkably reduced. Terrestrial mammals, including cows that are terrestrial relatives of whales, possess approximately 1,000 intact *OR* genes (Niimura et al. 2014; Niimura & Nei 2007), whereas minke whales (Mysticeti) possess only 60 intact *OR* genes, and 56 of these are included in class II (Kishida et al. in press). In addition, genomic analyses revealed that all modern mysticetes lack functional *OMACS* genes (Kishida et al. in press). Based on these findings, it appears that, although mysticetes have fully equipped olfactory systems, their OB lacks the D domain (Kishida et al. in press).

These molecular data suggest that mysticetes lack glomeruli on the dorsal side of their OB. In addition, because mysticetes possess a very small number of *OR* genes, it is expected that the number of glomeruli in their OB is also very small. However, no detailed study of the distribution and organization of glomeruli in mysticete OB has been reported to date. In this study, we provide the distribution of glomeruli in a mysticete, bowhead whale (*Balaena mysticetus* Linnaeus 1758) and test the "1 OR : 2 glomeruli" assumption in mysticetes.

Materials and methods

Tissues of bowhead whales were sampled under NOAA/NMFS permit 814-1899, and the preparation of tissue sections (thickness: 6 um) was described previously (Thewissen et al. 2011). Glomeruli are labeled by the expression of olfactory marker protein (OMP) (Danciger et al. 1989; Smith et al. 1991). The ImmunoCruz goat ABC staining system (Santa Cruz Biotechnology, Inc., cat no. sc-2023) and a rabbit polyclonal anti-OMP antibody (Santa Cruz Biotechnology, Inc., cat no. sc-67219) were used for immunohistochemistry, following the standard protocol attached to the ABC staining system kit. Antibody dilution was 1:150. The DAB-stained sections were counterstained with thionin, and then mounted on permanent slides. The number of glomeruli on each slide was counted manually, as shown in Supplementary Figs. S1, S2, S3, S4 and S5. In order to reconstruct a 3D image of the OB, horizontal sections of the whole OB of a bowhead whale (specimen no. 09B14) were prepared and every 5th slice was stained with thionin, mounted on permanent slides and photographed. Using AMIRA software (FEI Visualization Science Group) ver. 5.4.1, these images were aligned with manual adjustments, and 3D reconstructed. A STL-formatted 3D bowhead whale OB image thus obtained is available upon request (Supplemental Data S1, file size: 509MB).

Results and discussion

Fig. 1 and Supplementary Data S1 show the distribution patterns of glomeruli of the OB of bowhead whales. The shape of whale OB is not similar to that of terrestrial mammals such as mice. The olfactory ventricle is wide open dorsally and few glomeruli are found in the dorsal side of whale OB. This is in accordance with our genomic findings that modern mysticetes lack receptors and marker proteins that are specific to the D domain of the OB (Kishida et al. in press). We conclude that, from both genomic and morphological points of view, mysticete OB lacks the D domain.

In order to test the "1 OR: 2 glomeruli" assumption in mysticetes, we counted the numbers of glomeruli on five coronal sections, as shown in Fig. 2. We do observe that the numbers of glomeruli shown in Fig. 2 is likely to be an underestimate of the actual number because some glomeruli cannot be discriminated clearly and are not counted. Generally, four coronal sections were mounted in one slide, and the thickness of each section is 6um. It is estimated that 10 slides, containing 40 sections, correspond to 240um. Because the largest glomeruli are less than 240um in diameters (Supplementary Figs. S1, S2, S3, S4, S5 (coronal sections) and S6 (a horizontal section)), it can be expected that new glomeruli should appear at most every 10th slide. Therefore, we roughly estimated the number of glomeruli in approximately every 10th slide (Supplementary Table S1). Surprisingly, the bowhead whale OB is estimated to include approximately 4,000 glomeruli, a number much higher than that of mice (1,600-1,800) (Royet et al. 1988; Taniguchi et al. 2003). Given our method, this is an underestimate as explained above, and because slides at the rear of slide 518 were not taken into account.

Whole genome sequence data are required to obtain the repertoire of *OR* genes , but no bowhead whale genome assembly has been reported to date. Therefore, it is impossible to reveal the accurate number of *OR* genes in the bowhead whale genomes. Godfrey et al. showed that the olfactory anatomy of modern minke whale, whose whole genome assemblies have been reported (Kishida et al. in press; Yim et al. 2014), resembles that of late Eocene archaeocetes (Godfrey et al. 2013), suggesting that minke whales may be used as a model taxon for olfactory capabilities of all modern mysticetes. In addition, previous PCR-based studies suggest that bowhead whales and minke whales possess similar *OR* gene repertoires (Kishida et al. 2007; Thewissen et al. 2011). Minke whales are reported to possess 60 intact *OR* genes (Kishida et al. in press; Yim et al. 2014), and we assume that bowhead whales also possess approx. 60 *OR* genes, much less than the number of glomeruli in their OB. At least, the number of OR genes in bowhead whale genome should be much less than that in cow genome (~1,000). In any case, it is concluded that the “1 OR : 2 glomeruli” rule is not applicable in bowhead whales.

Humans are also reported to possess higher numbers of glomeruli (3,000-9,000) than the number of *OR* genes (350) (Maresh et al. 2008), similar to the case of whales. Both humans and whales are known to have reduced their *OR* gene repertoires profoundly in their evolutionary pathways (Kishida et al. in press; Matsui et al. 2010). It is possible that, in whales and humans, the evolutionary decline in glomerulus numbers proceeds at a slower rate than the decline of *OR* genes, and that this cause the aberrant ratio. Following this explanation, the ancestors of both whales and humans are expected to have a ratio of numbers of OR genes to glomeruli that is greater than 0.5. However, cows, the terrestrial relatives of whales, possess approximately 1,000 *OR* genes (Niimura & Nei 2007), and the whale ancestors are also expected to possess ~1,000 *OR* genes, a much lower number than the number of glomeruli in whale OB. Similarly, the last common ancestors of all modern primates have been estimated to possess 585 *OR* genes (Matsui et al. 2010), a much lower number than the number of glomeruli in human OB. We speculate that the “1 OR : 2 glomeruli” rule is applicable in mice or even in all rodents, but that it fails for other taxa.

Conclusion

Our results showed that bowhead whale OB lacks glomeruli on the dorsal side, in accordance with the molecular data that all modern mysticetes lack receptors and marker proteins that are specific to the D domain of the OB.

There is much larger number of glomeruli in the bowhead OB than expected from the number of *OR* genes, indicating that the “1 OR: 2 glomeruli” rule is not applicable to mysticetes.

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Figure legends

Figure 1.

Olfactory bulb of the bowhead whale brain.

a. Dorsal view of the left and right OBs of bowhead whale (specimen no. 09B14). Scale bar, 10mm.

b. Diagram of the dorsal and ventral view of the bowhead whale right OB. Coronal section (**c**) was cut at approximately the red dashed line.

c. Coronal section of right olfactory bulb of whale (specimen no. 09B11, section195c). Glomeruli were stained with DAB using anti-OMP antibody, and the whole tissue was counterstained with thionin. D, dorsal; L, lateral; M, medial; V, ventral. Scale bar, 1mm.

d. A schematic view of the distribution of glomeruli of the coronal section of the whale OB.

Figure 2.

Nos. of glomeruli in five coronal sections investigated in this study. Sections were cut at approximately the red dashed lines. Detail pictures of the sections are available as Supplementary Figures S1 (slide no. 32), S2 (slide no. 143), S3 (slide no. 195), S4 (slide no. 391) and S5 (slide no. 518).

Supplementary materials

Supplementary Data S1

A 3D image of a bowhead whale olfactory bulb provided as a STL format file (binary STL).

Supplementary Figure S1

A coronal section of the OB of bowhead whale 09B11 (section 32). Glomeruli are labeled with anti-OMP antibody, and are indicated with arrows. Scale bar, 1000um.

Supplementary Figure S2

A coronal section of the OB of bowhead whale 09B11 (section 143). Glomeruli are labeled with anti-OMP antibody, and are indicated with arrows. Scale bar, 1000um.

Supplementary Figure S3

A coronal section of the OB of bowhead whale 09B11 (section 195). Glomeruli are labeled with anti-OMP antibody, and are indicated with arrows. Scale bar, 1000um.

Supplementary Figure S4

A coronal section of the OB of bowhead whale 09B11 (section 391). Glomeruli are labeled with anti-OMP antibody, and are indicated with arrows. Scale bar, 1000um.

Supplementary Figure S5

A coronal section of the OB of bowhead whale 09B11 (section 518). Glomeruli are labeled with anti-OMP antibody, and are indicated with arrows. Scale bar, 1000um.

Supplementary Figure S6

A horizontal section of the OB of bowhead whale 09B14 (section 134). Glomeruli are labeled with anti-OMP antibody. Scale bar, 1000um. Left, anterior; right, posterior.

Supplementary Table S1

Number of glomeruli on approx. every 10th slide. Nos. of glomeruli with slide nos. in parentheses are estimated by taking an average between the glomeruli-counted sections in front and in the rear.

Figure 1(on next page)

Figure 1

Olfactory bulb of the bowhead whale brain. **a**. Dorsal view of the left and right OBs of bowhead whale (specimen no. 09B14). Scale bar, 10mm. b . Diagram of the dorsal and ventral view of the bowhead whale right OB. Coronal section (**c**) was cut at approximately the red dashed line.**c**. Coronal section of right olfactory bulb of whale (specimen no. 09B11,section195c). Glomeruli were stained with DAB using anti-OMP antibody, and the whole tissue was counterstained with thionin. D, dorsal; L, lateral; M, medial; V, ventral. Scale bar, 1mm.**d**. A schematic view of the distribution of glomeruli of the coronal section of the whale OB.

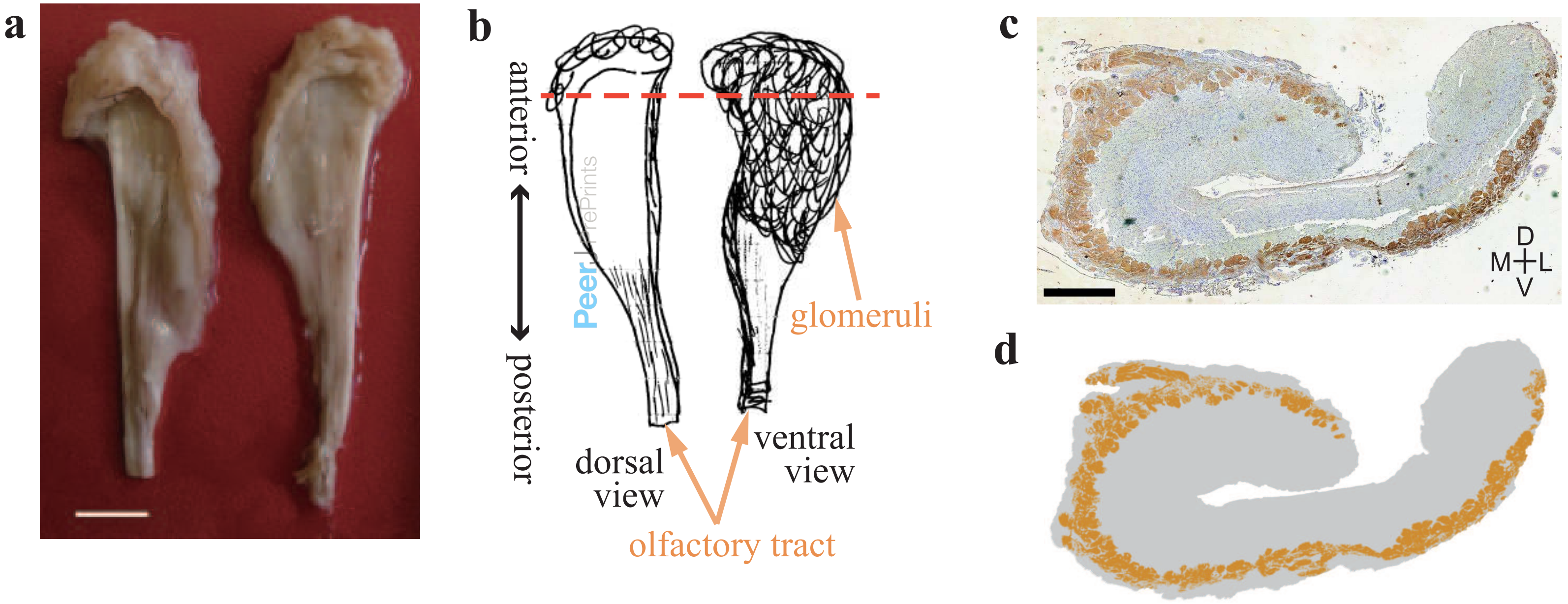
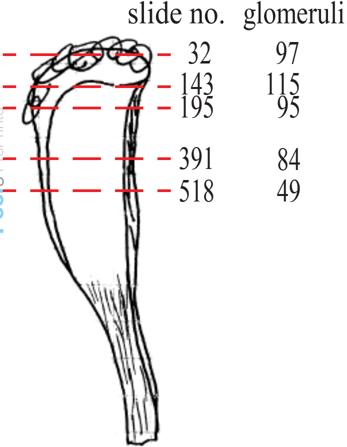

a
b
anterior
posterior
glomeruli
dorsal view
ventral view
olfactory tract
c
D
M
L
V
d

Figure 2(on next page)

Figure 2

Nos. of glomeruli in five coronal sections investigated in this study. Sections were cut at approximately the red dashed lines. Detail pictures of the sections are available as Supplementary Figures S1 (slide no. 32), S2 (slide no. 143), S3 (slide no. 195), S4 (slide no. 391) and S5 (slide no. 518).



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