Histological Properties of Main and Accessory Olfactory Bulbs in the Common Hippopotamus

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RUNNING HEAD: Hippopotamus olfactory bulb

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ABSTRACT

The olfactory system of mammals comprises a main olfactory system that detects hundreds of odorants and a vomeronasal system that detects specific chemicals such as pheromones. The main (MOB) and accessory (AOB) olfactory bulbs are the respective primary centers of the main olfactory and vomeronasal systems. Most mammals including artiodactyls possess the large MOB and comparatively small AOB, whereas most cetaceans lack the olfactory bulb. The common hippopotamus (*Hippopotamus amphibius*) is semiaquatic and belongs to the order Cetartiodactyla, family Hippopotamidae, which seems to be the closest extant family to cetaceans. The present study evaluates the significance of the olfactory system in the hippopotamus by histologically analyzing the MOB and AOB of a male common hippopotamus. The MOB comprised six layers (olfactory nerve, glomerular, external plexiform, mitral cell, internal plexiform and granule cell layers), and the AOB comprised vomeronasal nerve, glomerular, plexiform and granule cell layers. The MOB contained mitral cells and tufted cells, and the AOB possessed mitral/tufted cells. These histological features of the MOB and AOB were similar to those in most artiodactyls. All glomeruli in the AOB were positive for anti-$G_{\alpha i2}$, but weakly-positive for anti-$G_{\alpha o}$, suggesting that the hippopotamus vomeronasal system expresses vomeronasal type 1 receptors with high affinity for volatile compounds. These findings suggest that the olfactory system of the hippopotamus is as

well developed as that of other artiodactyl species and that the hippopotamus might depend on its olfactory system for terrestrial social communication.

List of abbreviations	
AOB	accessory olfactory bulb
EPL	external plexiform layer
$G_{\alpha i2}$	G protein alpha-i2 subunit
$G_{\alpha o}$	G protein alpha-o subunit
$G_{\alpha olf}$	G protein alpha-olf subunit
GCL	granule cell layer
GL	glomerular layer
IPL	internal plexiform layer
MCL	mitral cell layer
MOB	main olfactory bulb
OMP	olfactory marker protein
ONL	olfactory nerve layer
OR	olfactory receptor
PL	plexiform layer
V1R	vomeronasal type 1 receptors
V2R	vomeronasal type 2 receptors
VNL	vomeronasal nerve layer

INTRODUCTION

Mammals have a main olfactory system that detects hundreds of odorants and a vomeronasal system that detects specific chemicals such as pheromones. The olfactory bulb is the primary center of the olfactory system, and it consists of a main olfactory bulb (MOB) and an accessory olfactory bulb (AOB), that respectively receive projections from receptor cells of the main olfactory and vomeronasal systems. Axons of these receptor cells establish synapses with secondary olfactory neurons to form glomeruli in the olfactory bulb. Secondary olfactory neurons in the MOB mainly comprise mitral cells and tufted cells, whereas those in the AOB comprise a single cell type (mitral/tufted cells). The size and laminar structure of the olfactory bulb, especially the AOB, vary among species and seem to be associated with their habitats [Allison, 1952; Meisami and Bhatnagar, 1998]. Most mammals possess the large MOB and comparatively small AOB, but the olfactory bulb is very small and insignificant in some species such as marine mammals [Allison, 1952; Meisami and Bhatnagar, 1998].

The MOB of most mammals histologically consists of the following six layers: the olfactory nerve, glomerular, external plexiform, mitral cell, internal plexiform and granule cell layers. The AOB of rodents also consists of six layers, namely the vomeronasal nerve, glomerular, external plexiform, mitral/tufted cell, internal plexiform and granule cell layers [Meisami and Bhatnagar, 1998]. On the other hand, the external plexiform, mitral/tufted cell and internal plexiform layers in the AOB of artiodactyls are not individually separated because of the dispersion of mitral/tufted cells [Salazar et al., 2003; Mogi et al., 2007; Park et al., 2014], and thus the AOB is generally divided into four layers.

The odorant receptors are G protein-coupled, and they generally belong to four families

that are differentially expressed in the olfactory system among variously animal species. The families are olfactory receptors (OR) and trace amine-associated receptors coupled to the G protein alpha-olf subunit ($G_{\alpha olf}$) and vomeronasal types 1 (V1R) and 2 (V2R) receptors that are respectively coupled to the G protein alpha-i2 ($G_{\alpha i2}$) and G protein alpha-o ($G_{\alpha o}$) subunits [Jones and Reed, 1989; Berghard and Buck, 1996]. The V1R and V2R generally have high affinity for volatile and non-volatile substances, respectively [Peele et al., 2003; Kimoto et al., 2005], and the immunohistochemical finding of anti-G protein alpha subunits in the glomerular layer of AOB might reflect expressed receptor families and the volatile or non-volatile nature of compounds received by the vomeronasal system.

Hippopotami are semiaquatic, living in water during the day and on land during the night [Laws, 2012]. They belong to the Cetartiodactyla, family Hippopotamidae, which might be the closest extant family to cetaceans [Ursing and Arnason, 1998; Nikaido et al., 1999; Geisler and Theodor, 2009; Zhou et al., 2011; Gatesy et al., 2013]. Gatesy et al. [2013] suggested that a Hippopotamidae-Cetacea clade separated from a ruminant clade approximately 60 million years ago and that the ancestors of hippopotami and cetaceans divided 55 million years ago. Most artiodactyls within the Cetartiodactyla possess a vomeronasal system and a developed main olfactory system, whereas the vomeronasal system is lost and the main olfactory system is degraded in cetaceans [Meisami and Bhatnagar, 1998; Kishida et al., 2007, 2015a, 2015b; McGowen et al., 2008; Thewissen et al., 2011]. Baleen whales lack the dorsal domain of the olfactory bulb [Kishida et al., 2015b], and the olfactory bulb is absent in toothed whales [Oelschläger et al., 2010]. Artiodactyls express thousands of OR and some V1R (cows, intact 40 genes), but not functional V2R [Shi & Zhang, 2007], whereas cetaceans possess about 60 ORs and very

few V1R genes (mink whales, intact two genes; bottlenose dolphins, one gene) and lack V2Rs [Yim et al., 2014; Kishida et al., 2015a]. Unlike cetaceans, a pair of large olfactory bulbs [Garrod, 1880; Butti et al., 2014] and peripheral vomeronasal organs [Estes, 1991; personal observation] have been recognized in hippopotami at the gross anatomical level, but the presence of the AOB and the histological features of the olfactory bulb have remained unknown. Here, we aimed to determine the significance and functionality of the hippopotamus olfactory system by histologically and immunohistochemically analyzing the MOB and AOB of a male common hippopotamus (*Hippopotamus amphibius*).

MATERIALS AND METHODS

Animal

The brain of a 47-year-old male common hippopotamus (Japanese regional studbook number 85) that died on May, 2016 (Obihiro Zoo, Obihiro, Japan) was removed at necropsy, and then the olfactory bulb was sampled. The organ was free of pathological findings except age-related lipofuscin granules. This study proceeded according to the Regulations on Management and Operation of Animal Experiments, and the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine (No. 28-51) was notified of the experimental protocol.

Antibodies

The primary antibodies comprised polyclonal rabbit antibodies against olfactory marker protein (OMP; sc-67219), $G_{\alpha olf}$ (sc-383), and $G_{\alpha o}$ (sc-387) (all from Santa Cruz Biotech., Santa Cruz, CA, USA), and, $G_{\alpha i2}$ (ab20392, Abcam, Cambridge, MA, USA). The secondary antibody was biotinylated polyclonal goat antibody against rabbit IgG (BA-

1000; Vector Laboratories Inc., Burlingame, CA, USA).

Histological procedures

The paired olfactory bulb was fixed in Bouin's fluid and embedded in paraffin according to standard procedures. The right and left olfactory bulbs were cut frontally and sagittally, respectively, into 5-μm-thick sections, some of which were deparaffinized, stained with luxol fast blue/cresyl violet (staining of Klüver-Barrera) and histologically assessed using a Microphot-FX (Nikon, Tokyo, Japan) equipped with a Digital Sight DS-5M (Nikon). Other sections were processed for immunohistochemistry.

Immunohistochemistry

Deparaffinized, rehydrated sections were incubated with 0.3% H_2O_2 in methanol to eliminate endogenous peroxidase. The sections were rinsed in phosphate buffered saline (0.01 M, pH 7.4) and incubated with 3% normal goat serum to block nonspecific binding. The sections were incubated with anti-OMP (2.0 μg/mL), anti-$G_{\alpha olf/s}$ (0.4 μg/mL), anti-$G_{\alpha i2}$ (2.5 μg/mL) or anti-$G_{\alpha o}$ (2.0 μg/mL) antibodies overnight at 4°C. The sections were rinsed, incubated with biotinylated secondary antibody (7.5 μg/ml) for 1 h, reacted with the avidin-biotin complex reagent (PK-6100, Vector) and colored with Tris-HCl buffer containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.006% H_2O_2. Control sections were performed by the use of normal goat serum to replace primary antibodies.

RESULTS

Topographical features of the olfactory bulb

The olfactory bulb comprised a pair of spherical protuberances (rostrocaudal,

dorsoventral and mediolateral length, ~30-, 20- and 10-mm, respectively) from the brain that were located at the anterior end of the telencephalon (Fig. 1A). A laminar structure contained the olfactory ventricle (Fig. 1B, C, E, F). The MOB occupied most of the olfactory bulb, and the AOB was identified as a relatively small hemispherical structure (rostrocaudal length, ~ 3 mm) located at the dorsocaudal and medial parts of the olfactory bulb (Fig. 1D, G).

Histological features of the MOB

The MOB comprised olfactory nerve, glomerular, external plexiform, mitral cell, internal plexiform and granule cell layers (Fig. 1C, E, F, 2A). Round or oval glomeruli (diameter range: 50-300 μm) were surrounded by many small periglomerular cells (diameter ~5 μm) that formed semilunar shells (Fig. 2B). Some tufted cells (long axis ~30 μm; Fig. 2C) and interneurons were scattered in the external plexiform layer, and mitral cells (long axis ~30 μm; Fig. 2D) were arranged to form the mitral cell layer.

Histological and immunohistochemical features of the AOB

The AOB comprised vomeronasal nerve, glomerular, plexiform and granule cell layers (Fig. 1F, 2E). The lateral olfactory tract was located below the granule cell layer (Fig. 1F, 2E). Glomeruli (diameter range: 50-300 μm) appeared as aggregated fibers (Fig. 2F), and periglomerular cells (diameter ~5 μm) roughly surrounding the glomeruli. The mitral/tufted cells (long axis ~20 μm) were scattered in plexiform layer (Fig. 2G). The vomeronasal nerve layer and the glomeruli in the AOB were both positive for anti-OMP (Fig. 3A, B) and -$G_{\alpha i2}$ (Fig. 3G, H), but negative for anti-$G_{\alpha olf}$ (Fig. 3D, E). Anti-$G_{\alpha o}$ obviously reacted to plexiform and granule cell layers, but weakly and uniformly to the

vomeronasal nerve and glomerular layers (Fig. 3J, K). On the other hand, glomeruli in the MOB were positive for anti-OMP, -$G_{\alpha olf}$ and -$G_{\alpha o,}$ but negative for anti-$G_{\alpha i2}$ (Fig. 3C, F, I, L). No specific stainings were observed in the control section (Fig. 3M-O).

DISCUSSION

The present findings indicated that layer structures and cellular components of the MOB and AOB in the hippopotamus are similar to those of the MOB and AOB of other artiodactyl species [Allison, 1952; Meisami and Bhatnagar, 1998; Salazar et al., 2003; Mogi et al., 2007; Park et al., 2014]. The MOB and AOB glomeruli were positive for anti-OMP, a protein expressed in mature olfactory neurons [Margolis, 1972], supporting the notion that olfactory and vomeronasal receptor cells project to the MOB and AOB, respectively. Considering the morphologically large olfactory bulb in the hippopotamus [Garrod, 1880; Butti et al., 2014] together with the present findings, we suggest that the hippopotamus uses the olfactory system to detect chemical substances in the external environment like other artiodactyls.

Recent genetic [McGowen et al., 2008; Kishida et al., 2007, 2015a] and morphological [Thewissen et al., 2011; Kishida et al., 2015b] studies have found a depressed olfactory system in aquatic mammals, indicating a reduced need for this system in water, like traditional theories [Allison, 1952; Meisami and Bhatnagar, 1998]. Hippopotami are semiaquatic and spend the daytime in rivers and lakes. Many genetic analyses have shown that the family Hippopotamidae is close to cetaceans [Ursing and Arnason, 1998; Nikaido et al., 1999; Geisler and Theodor, 2009; Zhou et al., 2011; Gatesy et al., 2013]. The layer structure of the neocortex (without layer IV) in hippopotami is similar to that in cetaceans, but not artiodactyls [Butti et al., 2011, 2014]. However, we showed that the hippopotamus

possesses a developed MOB and AOB like other artiodactyls, suggesting that they use the olfactory system when on land.

The olfactory bulb might be undeveloped in some amphibious mammals such as pinnipeds [Montie et al., 2009], otters [Radinsky, 1968] and platypus [Ashwell, 2013]. However, the ratio of the olfactory bulb volume to total brain volume is similar between beavers [Müller-Schwarze & Sun, 2003] and terrestrial rodents, indicating that beavers also have a sensitive olfactory system that is comparable to that of terrestrials. Considering these reports together with the present findings, the degree of dependence on olfaction in semiaquatic mammals varies and seems to reflect their lifestyles.

Hippopotamus MOB glomeruli were positive for both anti-$G_{\alpha olf}$ and anti-$G_{\alpha o}$, like other mammals such as rats [Shinohara et al., 1992], mice [Wekesa and Anholt, 1999], wallaby [Schneider et al., 2012] and goats [Takigami et al., 2000]. $G_{\alpha o}$ is involved in synaptic functions and cell-to-cell contacts in the nervous system [Jiang and Bajpayee, 2009], and Choi et al. [2016] reported that the OB development and differentiation of dopaminergic neurons in the MOB are inhibited in $G_{\alpha o}$-deficient mice. In addition, common ancestors of all modern artiodactyls lost intact V2R genes [Shi and Zhang, 2007]. Therefore, $G_{\alpha o}$ appears to play a role in neuronal activity rather than as an odorant receptor-binding protein in the main olfactory system, and hippopotami seem to exclusively express OR-$G_{\alpha olf}$ in the main olfactory system like many other mammals.

Most species, that possess only V1R genes such as Laurasiatheria [Shi & Zhang, 2007], have only AOB glomeruli that are intensely positive for anti-$G_{\alpha i2}$ [Takigami et al., 2000; 2004], whereas the AOB glomeruli of animal groups such as Squamata [Brykczynska et al., 2013] that possess mainly V2R genes are positive for anti-$G_{\alpha o}$, but negative for anti-$G_{\alpha i2}$ [Kondoh et al., 2013]. In contrast, most rodents possess equal amounts of both V1R

and V2R genes [Shi & Zhang, 2007] and two types of glomeruli in the AOB. Glomeruli at the anterior half of the glomerular layer in the mouse AOB that receives axons derived from V1R neurons are intensely and weakly positive for anti-$G_{\alpha i2}$ and anti-$G_{\alpha o}$, respectively, whereas posterior glomeruli that receive axons of V2R neurons are intensely-positive for anti-$G_{\alpha o}$ but negative for anti-$G_{\alpha i2}$ [Jia & Halpern, 1996]. All glomeruli in the AOB of the hippopotamus were intensely positive for anti-$G_{\alpha i2}$ (see also supplemental Fig. 1) and weakly positive for anti-$G_{\alpha o}$, suggesting that the hippopotamus vomeronasal system expresses exclusively V1R-$G_{\alpha i2}$, like many other Laurasiatheria species including goats [Takigami et al., 2000] and cows [Shi and Zhang, 2007].

Shi & Zhang (2007) indicated that V1Rs of vertebrates are divided into three major clades, and that mammals possess clade 1 whereas fishes possess clades 2 and 3 V1Rs. Mammalian V1R generally have high affinity for volatile compounds [Peele et al., 2003], although fish V1R might detect the water-soluble substances. The vomeronasal system of the hippopotamus might be adapted to function under water [Estes, 1991], but Zapico (1999) has described a male common hippopotamus exhibiting flehmen behavior, which transfers pheromones into the vomeronasal organ, on land and waterfront areas. Our findings support the notion that the hippopotamus detects volatile pheromones while on land using a vomeronasal system that expresses V1R-$G_{\alpha i2}$.

The present histological findings, like previous morphological findings [Garrod, 1880; Butti et al., 2014], suggested that the olfactory system of the hippopotamus is as well-developed as that of other artiodactyl species. Hippopotami shower dung/urine [Estes, 1991], walk along trails marked by dung piles at night [Laws, 2012] and exhibit flehmen behavior on land [Zapico, 1999]. Our immunohistochemical findings notably suggested that the vomeronasal system of the hippopotamus detects volatile species-specific

compounds. Although the Hippopotamidae is the closest extant family to Cetacea [Ursing and Arnason, 1998; Geisler and Theodor, 2009; Zhou et al., 2011; Gatesy et al., 2013], which has an essentially insignificant olfactory system [Allison, 1952; Meisami and Bhatnagar, 1998], the olfactory system of the hippopotamus might be critical for life and social communication, especially when on land.

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REFERENCES

Allison AC (1953): The morphology of the olfactory system in the vertebrates. Biol Rev 28:195–244.

Ashwell KWS (2013): Chemical senses: olfactory and gustatory systems; in Neurobiology of Monotremes Brain Evolution in Our Distant Mammalian Cousins. Collingwood, CSIRO Publishing, pp 235–250.

Berghard A, Buck LB (1996): Sensory transduction in vomeronasal neurons: evidence for $G_{\alpha o}$, $G_{\alpha i2}$, and adenylyl cyclase II as major components of a pheromone signaling cascade. J Neurosci 16:909–918.

Brykczynska U, Tzika AC, Rodriguez I, Milinkovitch MC (2013): Contrasted evolution of the vomeronasal receptor repertoires in mammals and squamate reptiles. Genome Biol Evol 5:389–401.

Butti C, Raghanti MA, Sherwood CC, Hof PR (2011): The neocortex of cetaceans:

cytoarchitecture and comparison with other aquatic and terrestrial species. Ann NY Acad Sci 1225:47–58.

Butti C, Ewan Fordyce R, Ann Raghanti M, Gu X, Bonar CJ, Wicinski BA, Wong EW, Roman J, Brake A, Eaves E, Spocter MA, Tang CY, Jacobs B, Sherwood CC, Hof PR (2014): The Cerebral Cortex of the Pygmy Hippopotamus, *Hexaprotodon liberiensis* (Cetartiodactyla, Hippopotamidae): MRI, Cytoarchitecture, and Neuronal Morphology. Anat Rec (Hoboken) 297:670–700.

Estes RD (1991): Hippopotamuses Family Hippopotamidae; in The behavior guide to African mammals. Berkeley, University of California Press, pp 222–226.

Garrod AH (1880): On the brain and other parts of the hippopotamus (*H. amphibius*). Trans Zool Soc Lond 11:11–17.

Gatesy J, Geisler JH, Chang J, Buell C, Berta A, Meredith RW, Springer MS, McGowen MR (2013): A phylogenetic blueprint for a modern whale. Mol Phylogenet Evol 66:479–506.

Geisler JH, Theodor JM (2009): Hippopotamus and whale phylogeny. Nature 458:E1–4.

Jia C, Halpern M (1996): Subclasses of vomeronasal receptor neurons: differential expression of G proteins ($G_{i\alpha 2}$ and $G_{o\alpha}$) and segregated projections to the accessory olfactory bulb. Brain Res 719:117–128.

Jones DT, Reed RR (1989): Golf: an olfactory neuron specific-G protein involved in odorant signal transduction. Science 244:790–795.

Kimoto H, Haga S, Sato K, Touhara K (2005): Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. Nature 437:898–901.

Kishida T, Kubota S, Shirayama, Fukami H (2007): The olfactory receptor gene repertoires in secondary-adapted marine vertebrates: evidence for reduction of the functional

proportions in cetaceans. Biol Lett 3:428–430.

Kishida T, Thewissen J, Hayakawa T, Imai H, Agata K (2015a): Aquatic adaptation and the evolution of smell and taste in whales. Zoological Lett 1:9.

Kishida T, Thewissen J, Usip S, Suydam RS, George JC (2015b): Organization and distribution of glomeruli in the bowhead whale olfactory bulb. PeerJ 3:e897.

Kondoh D, Koshi K, Ono HK, Sasaki K, Nakamuta N, Taniguchi K (2013): Identification of G protein α subunits in the main olfactory system and vomeronasal system of the Japanese Striped snake, *Elaphe quadrivirgata*. J Vet Med Sci 75:381–385.

Ladinsky LB (1968): Evolution of somatic sensory specialization in otter brains. J Comp Neurol 134:495–505.

Laws RM (2012): Hippopotamus biology; in Blix AS (ed): Large animals and wide horizons: adventures of a biologist–The autobiography of Richard M. Laws. Part II. pp.130–137. http://www.spri.cam.ac.uk/resources/autobiographies/richardlaws/

Margolis FL (1972): A brain protein unique to the olfactory bulb. Proc Nat Acad Sci USA 69:1221–1224.

McGowen MR, Clark C, Gatesy J (2008): The vestigial olfactory receptor subgenome of odontocete whales: phylogenetic congruence between gene-tree reconciliation and supermatrix methods. Syst Biol 57:574–590.

Meisami E, Bhatnagar KP (1998): Structure and diversity in mammalian accessory olfactory bulb. Microsc Res Tech 43:476–499.

Mogi K, Sakurai K, Ichimaru T, Ohkura S, Mori Y, Okamura H (2007): Structure and chemical organization of the accessory olfactory bulb in the goat. Anat Rec (Hoboken) 290:301–310.

Montie EW, Pussini N, Schneider GE, Battey TW, Dennison S, Barakos J, Gulland F

(2009): Neuroanatomy and volumes of brain structures of a live California sea lion (*Zalophus californianus*) from magnetic resonance images. Anat Rec (Hoboken) 292:1523–1547.

Müller-Schwarze D, Sun L (2003): The organism; in The Beaver: Natural History of a Wetlands Engineer. Ithaca, Cornell University Press, pp 1–28.

Nikaido M, Rooney AP, Okada N (1999): Phylogenetic relationships among cetartiodactyls based on insertions of short and long interspersed elements: hippopotamuses are the closest extant relatives of whales. Proc Natl Acad Sci USA 96:10261–10266.

Oelschläger HHA, Ridgway SH, Knauth M (2010): Cetacean brain evolution: dwarf sperm whale (*Kogia sima*) and common dolphin (*Delphinus delphis*) – an investigation with high-resolution 3D MRI. Brain Behav Evol 75:33–62.

Park C, Ahn M, Lee JY, Lee S, Yun Y, Lim YK, Taniguchi K, Shin T (2014): A morphological study of the vomeronasal organ and the accessory olfactory bulb in the Korean roe deer, *Capreolus pygargus*. Acta Histochem 116:258–264.

Peele P, Salazar I, Mimmack M, Keverne EB, Brennan PA (2003): Low molecular weight constituents of male mouse urine mediate the pregnancy block effect and convey information about the identity of the mating male. Eur J Neurosci 18:622–628.

Salazar I, Lombardero M, Alemañ N, Sánchez Quinteiro P (2003): Development of the vomeronasal receptor epithelium and the accessory olfactory bulb in sheep. Microsc Res Tech 61:438–447.

Shi P, Zhang J (2007): Comparative genomic analysis identifies an evolutionary shift of vomeronasal receptor gene repertoires in the vertebrate transition from water to land. Genome Res 17:166–174.

Takigami S, Mori Y, Ichikawa M (2000): Projection pattern of vomeronasal neurons to the accessory olfactory bulb in goats. Chem Senses 25:387–393.

Takigami S, Mori Y, Tanioka Y, Ichikawa M (2004): Morphological evidence for two types of Mammalian vomeronasal system. Chem Senses 29:301–310.

Thewissen JGM, George J, Rosa C, Kishida T (2011): Olfaction and brain size in the bowhead whale (*Balaena mysticetus*). Mar Mamm Sci 27:282–294.

Ursing BM, Arnason U (1998): Analyses of mitochondrial genomes strongly support a hippopotamus-whale clade. Proc Biol Sci 265:2251–2255.

Yim HS, Cho YS, Guang X, Kang SG, Jeong JY, Cha SS, Oh HM, Lee JH, Yang EC, Kwon KK, Kim YJ, Kim TW, Kim W, Jeon JH, Kim SJ, Choi DH, Jho S, Kim HM, Ko J, Kim H, Shin YA, Jung HJ, Zheng Y, Wang Z, Chen Y, Chen M, Jiang A, Li E, Zhang S, Hou H, Kim TH, Yu L, Liu S, Ahn K, Cooper J, Park SG, Hong CP, Jin W, Kim HS, Park C, Lee K, Chun S, Morin PA, O'Brien SJ, Lee H, Kimura J, Moon DY, Manica A, Edwards J, Kim BC, Kim S, Wang J, Bhak J, Lee HS, Lee JH (2014): Minke whale genome and aquatic adaptation in cetaceans. Nat Genet 46:88–92.

Zapico TA (1999): First documentation of flehmen in a common hippopotamus (*Hippopotamus amphibius*). Zoo Biol 18:415–420.

Zhou X, Xu S, Yang Y, Zhou K, Yang G (2011): Phylogenomic analyses and improved resolution of Cetartiodactyla. Mol Phyl Evol 61:255–264.

FIGURE LEGENDS

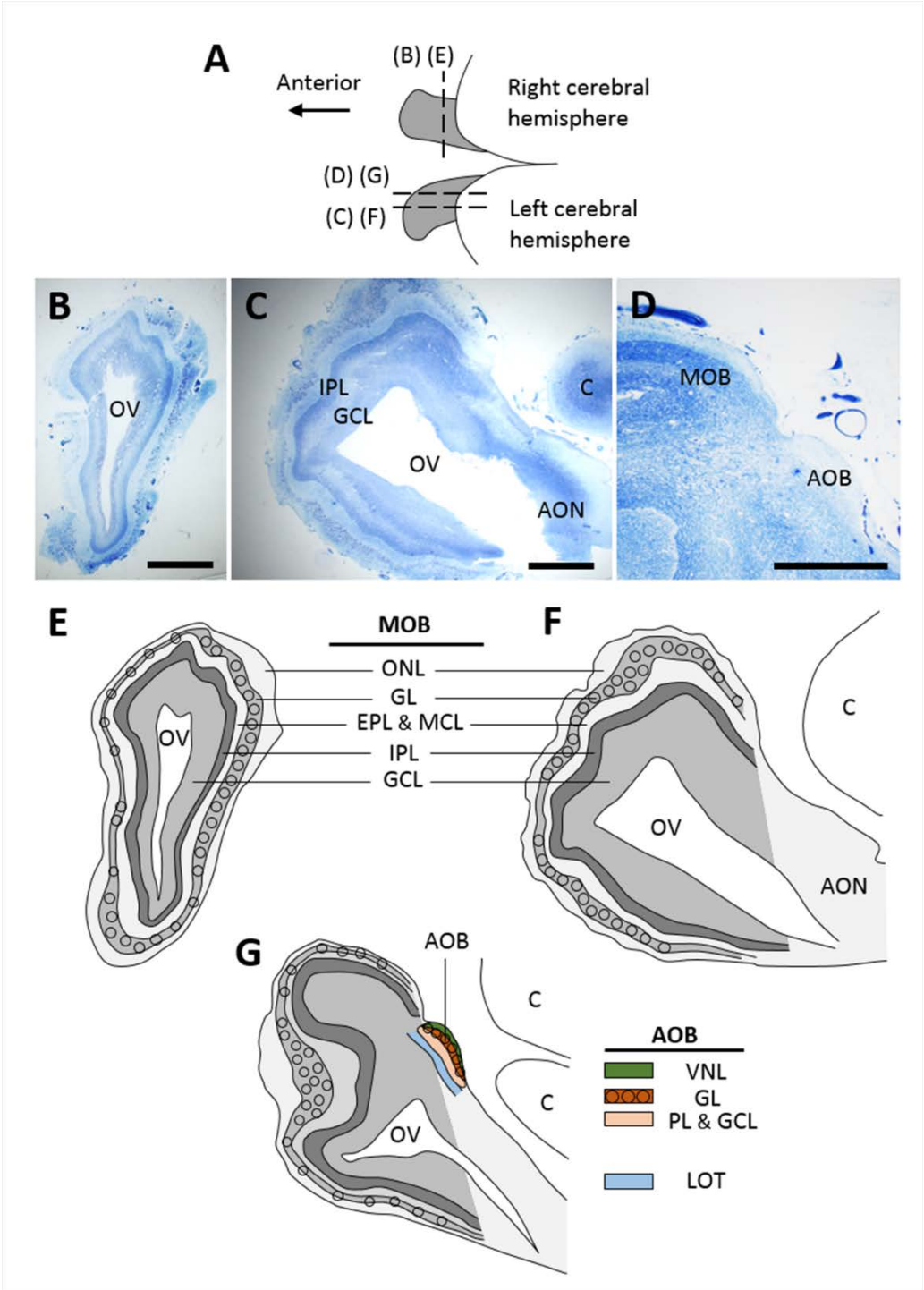
Fig. 1. Topographical features of main and accessory olfactory bulbs of the hippopotamus. **A**. Schema of dorsal view of anterior telencephalon including olfactory bulbs (gray regions). Dashed lines correspond to panels. **B–D**. Frontal (**B)** and sagittal **(C)** sections of MOB and sagittal section of AOB **(D)**. Left, medial; upper, dorsal in (**B**), and left, rostral; upper, dorsal in (**C** and **D**). Accessory olfactory bulb appears in more medial than sagittal sections (**C**), and panel (**D**) shows dorsocaudal part of olfactory bulb. **E–G**. Schemas corresponding to panels (**B–D**, respectively). AOB, accessory olfactory bulb; AON, anterior olfactory nuclei; C, frontal cortex; EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; IPL, internal plexiform layer; LOT, lateral olfactory tract; MCL, mitral cell layer; MOB, main olfactory bulb; ONL, olfactory nerve layer; OV, olfactory ventricle PL, plexiform layer; VNL, vomeronasal nerve layer. Features visualized using Klüver-Barrera stain. Black bars = 5 mm.

Fig. 2. Histological features of main and accessory olfactory bulbs of the hippopotamus. **A.** Layer structures of MOB. **B.** Glomeruli (*) and periglomerular cells (arrowheads) in GL of MOB. **C.** Tufted cells (arrows) in EPL of MOB. **D.** Mitral cells (arrows) in MCL of MOB. **E.** Layer structures of AOB. Left, rostral; upper, dorsal. **F.** Glomerulus (dashed circle) of AOB. **G.** Mitral/tufted cell (arrow) in PL of AOB. Abbreviations correspond to those in Fig. 1. Features visualized using Klüver-Barrera stain. Black bars = 500 (**A and E**), 50 (**F**) and 20 (**B–D and G**) μm.

Fig. 3. Immunohistochemical features of accessory and main olfactory bulb in the hippopotamus.

Immunoreactivity against anti-OMP (**A–C**), -$G_{\alpha olf}$ (**D–F**), -$G_{\alpha i2}$ (**G–I**) and -$G_{\alpha o}$ (**J–L**) and controls without primary antibodies (**M–O**). Whole AOB (**A, D, G, J, M**), single glomerulus of AOB (surrounded by arrowheads) (**B, E, H, K, N**) and single glomerulus of MOB (**C, F, I, L, O**). AOB, accessory olfactory bulb; GL, glomerular layer; MOB, main olfactory bulb. Black bars = 500 (**A**) and 50 (**B and C**) µm.

Supplemental Fig. 1. High magnifications of anti-OMP (**A**) and anti-$G_{\alpha i2}$ (**B**) immunoreactivity in GL of AOB, corresponding to panels (Fig. 3 **A and G**, respectively). Sixteen glomeruli (dashed circles) are positive for both anti-OMP and anti-$G_{\alpha i2}$.


A
Anterior
(B) (E)
Right cerebral hemisphere
(D) (G)
(C) (F)
Left cerebral hemisphere
B
OV
C
IPL
GCL
C
OV
AON
D
MOB
AOB
E
MOB
ONL
GL
EPL & MCL
IPL
GCL
OV
F
C
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AON
G
AOB
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C
OV
AOB
VNL
GL
PL & GCL
LOT

Fig. 1

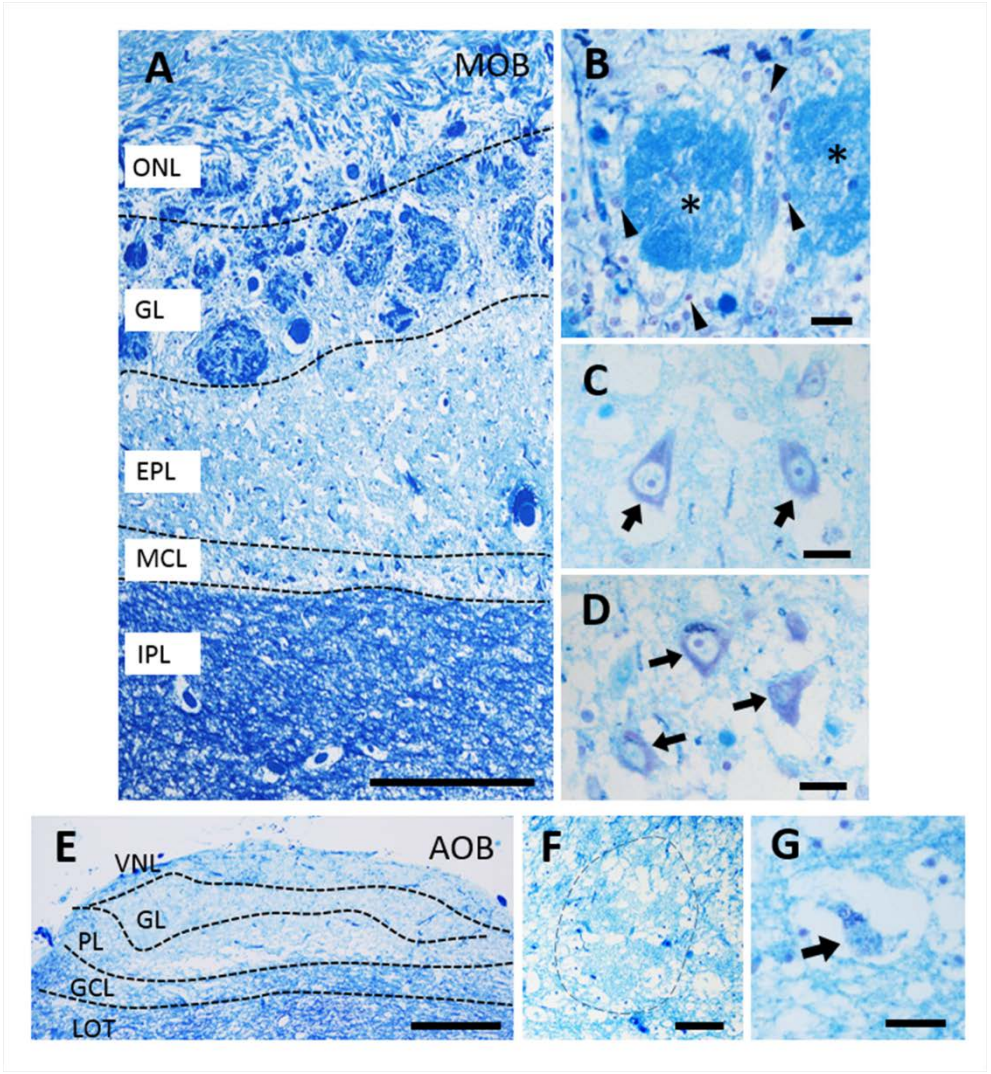


Fig. 2

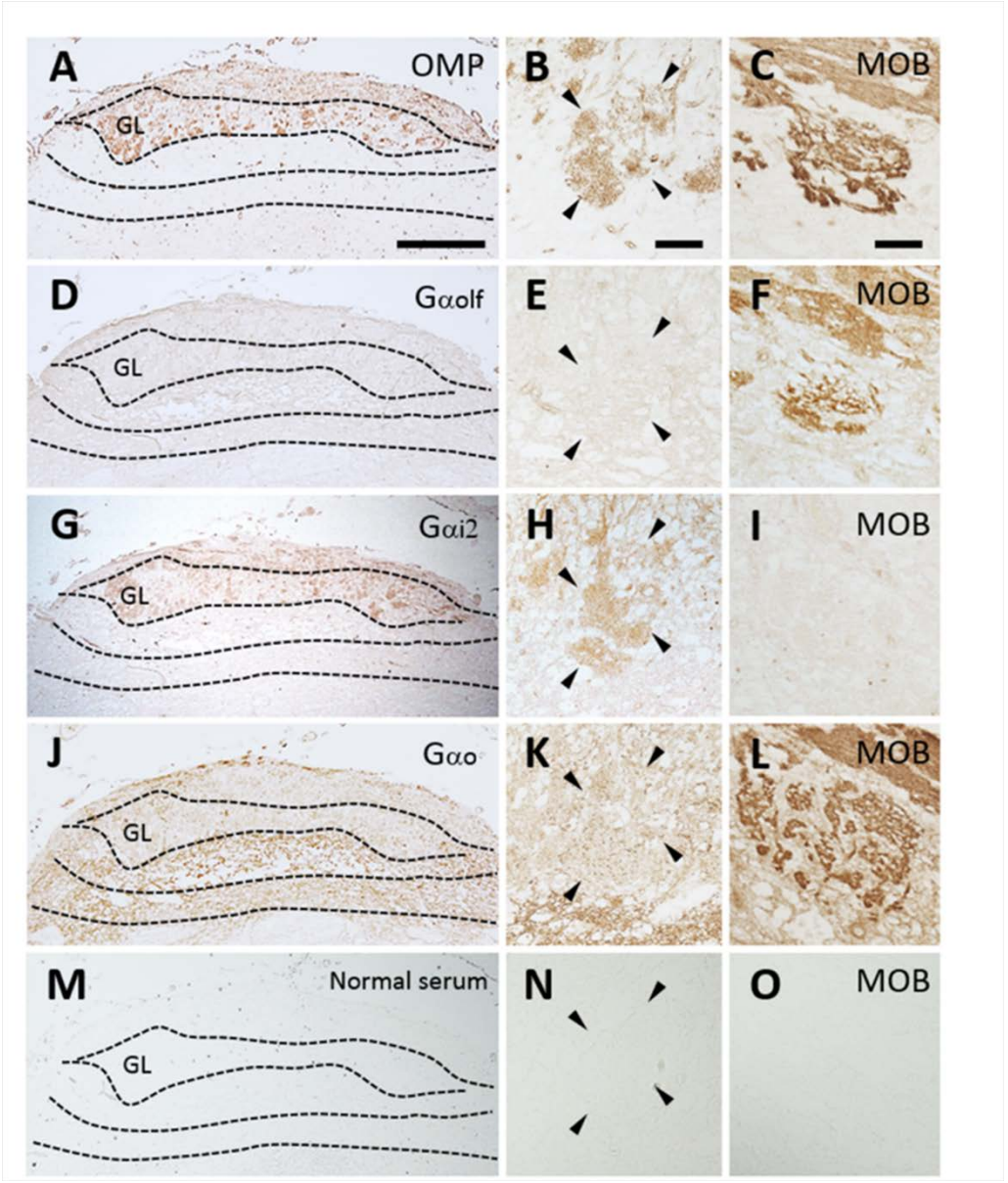
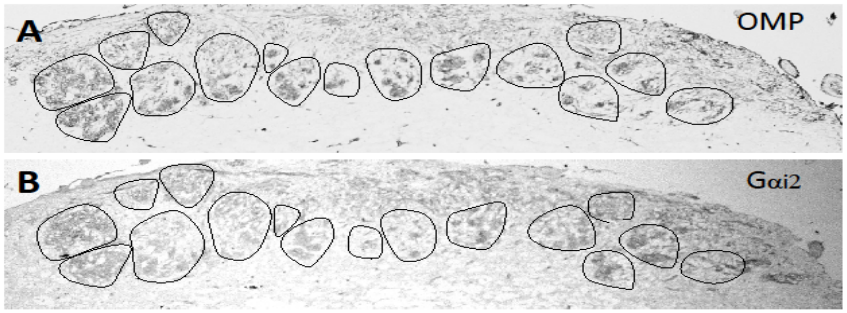

A
OMP
GL
B
C
MOB
D
Gαolf
GL
E
F
MOB
G
Gαi2
GL
H
I
MOB
J
Gαo
GL
K
L
MOB
M
Normal serum
GL
N
O
MOB

Fig. 3



Supplemental Fig. 1.
High magnifications of anti-OMP (A) and anti-Gαi2 (B) immunoreactivity in GL of AOB, corresponding to panels (Fig. 3 A and G, respectively). Sixteen glomeruli (dashed circles) are positive for both anti-OMP and anti-Gαi2.