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RESEARCH ARTICLE

Immunofluorescence evidence of melanotrophs in the pituitary of four odontocete species. An immunohistochemical study and a critical review of the literature

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SUMMARY

Cetaceans share peculiar features of their pituitary glands, with a complete separation of *pars distalis* and *pars nervosa* by a dural septum and the absence of an intermediate lobe and cleft. In most mammals the *pars intermedia* is the main source of circulating α-melanocyte stimulating hormone (α-MSH), derived from a large precursor called proopiomelanocortin (POMC), which also generates adrenocorticotropic hormone (ACTH) in the adenohypophysis. The lack of an intermediate lobe in cetaceans led us to investigate whether their glands are able to produce α-MSH, and if this hormone is secreted by a distinct population of melanotrophs or by corticotrophs in the *pars distalis*. Immunofluorescence evidences seem to support the first assumption, with ACTH-immunoreactive (-ir) elements rarely overlapping with α-MSH-ir ones. The discovery of a population of true melanotrophs in the hypophysis of some odontocetes underscores the need for further research on the melanocortin system of cetaceans

1. Introduction

It is well known that the mammalian pituitary derives from the fusion of two halves, one (the *pars nervosa*) growing from the floor of the diencephalon, the other (the *pars distalis*) from the roof of the pharynx (Wingstrand, 1966). In the pituitary gland of cetaceans, the *p. nervosa* (neurohypophysis) and the *p. distalis* (adenohypophysis) are completely separated by a dural septum (Arvy, 1971), while in most mammals they are juxtaposed. Besides, the intermediate lobe and cleft are missing, and both features are also observed in armadillos (Oldham, 1941), Indian elephants (Oboussier, 1948), dugongs (Fernand, 1951), pangolins (Herlant, 1958), and other non-mammalian species (Rahn and Painter, 1941; Saint-Girons, 1961; Takeuchi et al., 2003). In most other vertebrates, the *pars intermedia* is composed mainly of secreting cells called melanotrophs, since they are the principal source of circulating alpha-melanocyte-stimulating hormone (α-MSH), involved in the regulation of integumental pigmentation. This tridecapeptide is cleaved from adrenocorticotrophic hormone (ACTH), secreted by corticotrophs of the adenohypophysis, in turn derived by a very large precursor called pro-opiomelanocortin (POMC). The absence of an intermediate lobe in the above mentioned species led some investigators to think that α-MSH would not be produced by them, or that it would have a negligible role. Early bioassays on toads with pituitary homogenates of armadillos (*Dasypus novemcinctus*) (Oldham, 1938) demonstrated the opposite, showing that anterior lobes from this species contained a melanotropic agent. Bioassays on the skin of the lizard *Anolis carolinensis* indirectly revealed the presence of α-MSH in hypophyseal extracts of blue (*Balaenoptera musculus*) (Valsø, 1934), finback (*Balaenoptera physalus*) and sperm whales (*Physeter macrocephalus*) (Geiling, 1935). In particular, Valsø (1934) observed a melanin-dispersing activity of blue whale homogenates 20 times higher than that of human or bovine glands, and in particular a higher content of the melanotropic agent in the *pars distalis* than in the *pars tuberalis*. The same was found by Geiling et al. (1940) in the bottlenose (*Tursiops truncatus*) and the Atlantic spotted dolphin (*Stenella frontalis*). Oldham et al. (1940) further compared the content of a "melanophore-dispersing hormone" in different regions of the adenohypophysis of the armadillo and of three cetaceans, the bottlenose dolphin, the beluga (*Delphinapterus leucas*) and the fin whale. Notably, in all four species, the highest activity of the hormone was found in the antero-ventral region of the anterior lobe, while the lower activity was in the postero-dorsal (juxtaneural) one. As the authors noted, this is a remarkable observation since the latter would be the position of the intermediate lobe in most other mammals. Hoekstra and Burgers (1967) identified the chemical nature of melanotropins in extracts of fin whale hypophyses, and detected four different components, three of which very similar to known ones, i.e. α-, β-seryl-, and β-glutamyl-MSH, and a fourth unknown one, more abundant than

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the others. For at least 40 years the contribution to the knowledge about melanotropins in cetaceans was completely neglected, until recently when Cowan et al. (2008) provided an extensive immunohistochemical survey on the major pituitary hormones in several odontocete species. In that work, only a brief account for the bottlenose dolphin was provided, and in particular α-MSH-immunoreactive (-ir) cells were found abundant, faintly labeled and associated with follicles. The same authors claimed to have found a structure referable to an intermediate lobe, which they called "dorsal shoulder", although a clear characterization of the cytological or immunochemical differences with the rest of the *pars distalis* was missing. The aim of the present work was to evaluate the distribution of α-MSH-ir cells in the adenohypophysis of four odontocetes, and to understand if these cells could be true melanotrophs rather than a subset of corticotrophs. Since cetaceans are part of the order Cetartiodactyla, we analyzed also some porcine and ovine pituitaries for comparison.

2. Materials and methods

2.1. Specimen collection

The hypophyses from a total of 11 bottlenose dolphins (*Tursiops truncatus*), 2 Risso's dolphins (*Grampus griseus*), 2 striped dolphins (*Stenella coeruleoalba*) and 1 common dolphin (*Delphinus delphis*) were employed for immunostainings (Table 1). All animals were found stranded along the Italian coasts, except four bottlenose dolphins who lived and died in a controlled environment. All of the samples were provided by the Mediterranean Marine Mammal Tissue Bank (MMMTB), hosted by the University of Padova (http://www.mammiferimarini.sperivet.unipd.it/eng/index.htm). The age of wild specimens was estimated from the total body length or dentine layers counting. The age of the four captive bottlenose dolphins was determined by the existing documentation. All specimens were adults or subadults. The pituitaries were sampled during routine necropsies and were preserved in buffered formalin. The post-mortem interval prior to the necropsy varied from several hours to 1–2 days, and the carcasses were fresh to moderately fresh. The fixation period also varied considerably between 15 and 45 days. The pituitaries of two terrestrial species as well were sampled within few hours post-mortem, namely a sheep (*Ovis aries*) and a pig (*Sus scrofa*) that came from local commercial abattoirs. The glands were obtained according to the European Community Council directive 86/609/EEC, concerning animal welfare during the commercial slaughtering process. After fixation, the glands were rinsed overnight in running water to wash away the formalin, dehydrated in graded alcohols, embedded in paraffin and cut at the microtome in 5–6 μm thick sections.

Table 1
List of cetacean species used in this study, with given ID number of Marine Mammal Tissue Bank and date of necropsy.

Species	Bank ID	Date of necropsy
Delphinus delphis	5	30-10-2000
Grampus griseus	48	08-04-2003
	87	22-06-2005
Stenella coeruleoalba	49	16-09-2003
	214	18-05-2012
Tursiops truncatus	4	07-09-2000
	20	23-02-2002
	89	12-10-2005
	95	05-03-2006
	107	11-09-2006
	162	15-07-2006
	165	27-07-2009
	192	09-09-2010
	196	15-05-2011
	201	30-06-2011
	203	06-07-2011

2.2. Histological staining

The integrity and quality of the glands were evaluated by two different histological techniques: haematoxylin–eosin (HE) stain to observe the general architecture of the tissue and Slidder's trichrome stain (Mazzi, 1977) to discriminate among acidophilic, basophilic and chromophobic cells of the *pars distalis*.

2.3. Immunohistochemistry

We performed peroxidase-immunohistochemistry (IHC) following the avidin–biotin method. Briefly, after dewaxing, the sections were heated for 10 min at 120 °C in 10 mM citrate buffer pH 7.6 in an autoclave, for the antigen retrieval. The endogenous peroxidase activity was blocked by immersion in 10% H_2O_2 in methanol for 30 min at room temperature (RT), and the non-specific binding were prevented by incubating the sections in PBS pH 7.4 with 10% bovine serum albumin (BSA, Sigma–Aldrich, Poole, UK), 10% normal goat serum (NGS, Vector, Burlingame, CA) and 0.02% Triton-X100 (Sigma–Aldrich). The sections were then incubated overnight with the primary antibody at a dilution of 1:500 at 4 °C, rinsed in PBS and incubated with a biotinylated secondary antibody at a dilution of 1:200 for 1 h at RT. After incubation with avidin-biotin complex (Vector) for 1 h at RT, the sections were stained with a diaminobenzidine (DAB) solution (Vector) for less than 2 min until stain development, then dehydrated in graded alcohols, mounted and coverslipped. The immunostainings were directed against α-MSH, using a polyclonal antibody raised in rabbit (BP5037, Acris, Hiddenhausen, Germany), or against ACTH, using a monoclonal antibody raised in mouse against the middle region of the molecule (MBS312930, Emelca, Breda, The Netherlands), to avoid cross-reactions between the anti-ACTH antibody and α-MSH. In the first case, the secondary antibody was a biotinylated anti-rabbit IgG (Vector), and in the second case a biotinylated anti-mouse IgG (Vector), both raised in goat. Images were acquired with an Olympus BX-51 optical microscope using Olympus Cell-B software.

2.4. Immunofluorescence

The same primary antibodies used for peroxidase-immunohistochemistry were employed for single and double immunofluorescence (IF), following the same steps except for H_2O_2 treatment. The secondary antibody used for anti-α-MSH was a TRITC-conjugated swine anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:60 in PBS with 1% BSA, while for anti-ACTH a FITC-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) at a dilution of 1:200 was employed. For the double immunostaining, sections were first incubated with ACTH antiserum (dilution 1:50) overnight at 4 °C, then with its secondary antibody for 1 h at RT, then with α-MSH antiserum (dilution 1:500) for 4 h at RT and finally with its secondary antibody for 1 h at RT. The slides were coverslipped using Mowiol 4-88 (Polyscience Inc., Warrington, PA) added with p-phenilenediamine (PPD) as anti-fading agent, in a 9:1 ratio. Images were taken with a Leica TCS SP5 confocal microscope and processed with Image J 1.42q software (NIH, Bethesda, MD).

2.5. Specificity controls

To test the specificity of the primary antibodies, negative controls were evaluated both by omitting them and incubating the