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Simona Sacchini (simona.sacchini@ulpgc.es)
Universidad de las Palmas de Gran Canaria https://orcid.org/0000-0001-6493-1275
Josué Díaz
Texas A&M University College Station
Antonio Espinosa de los Monteros
Universidad de las Palmas de Gran Canaria
Yania Paz
Universidad de las Palmas de Gran Canaria
Yara Bernaldo de Quirós
Universidad de Las Palmas de Gran Canaria
Eva Sierra
Universidad de las Palmas de Gran Canaria
Manuel Arbelo
Universidad de las Palmas de Gran Canaria
Pedro Herráez
Universidad de Las Palmas de Gran Canaria - Campus de San Cristobal
Antonio Fernández
Universidad de las Palmas de Gran Canaria

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Amyloid-beta peptide and phosphorylated tau in the frontopolar cerebral cortex and in the cerebellum of toothed whales: aging *vs* hypoxia

Sacchini Simona [1], Díaz Josué [2,3] Espinosa de los Monteros Antonio [1], Paz Yania [1], Bernaldo de Quirós Yara [1], Sierra Eva [1], Arbelo Manuel [1*], Herráez Pedro [1], Fernández Antonio [1]*

[1] Veterinary Histology and Pathology, Institute of Animal Health, University of Las Palmas de Gran Canaria, Veterinary School, c/Transmontaña s/n, 35416, Arucas

[2] Laboratory of Wildlife Comparative Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil

[3] Texas A&M Veterinary Medical Diagnostic Laboratory, College Station, TX 77843, US

Correspondence:

simona.sacchini@ulpgc.es

manuel.arbelo@ulpgc.es

ABSTRACT

Background: Alzheimer's disease results from the interplay of multiple risk factors and their effects. Diving mammals may be routinely exposed to severe hypoxia when submerged. Among toothed whales, the beaked whales are particularly cryptic and routinely dive deeper than 1,000 m for about one hour in order to hunt deep-water squid and fish. We hypothesized that hypoxia could be a possible risk factor for neurodegenerative alterations in the central nervous system of beaked whales in particular, and toothed whales in general.

Results: Samples of frontal cerebral cortex and cerebellum were collected from nine animals, representing six different species of the suborder Odontoceti. Immunohistochemical analysis employed a monoclonal anti-β-amyloid (Aβ) and a polyclonal anti-neurofibrillary tangle (NFT) antibodies.

Six of nine (67%) animals showed positive immunolabeling for Aβ and/or NFT. The most striking findings were intranuclear Aβ immunopositivity in cerebral cortical neurons and NFT immunopositivity in cerebellar Purkinje neurons with granulovacuolar degeneration.

Herein, we present immunohistopathological findings classic of Alzheimer's and other neurodegenerative diseases in humans, in different brain locales of odontocete cetaceans. This study represents the first description of A β and NFT in the brain of beaked whales, adding also to the non-existent descriptions of GVD in the brain of non-experimental animals, being specifically the first report of granulovacuolar degeneration in the cerebellum. Our results further confirm the rarely reported intranuclear expression of A β.

Conclusions: These findings could be linked to hypoxic phenomena, as they were more extensive in the brains of beaked whales, and not only in aged individuals. Therefore, a novel hypothesis linking hypoxia and neurodegeneration microscopic hallmarks in cetaceans is proposed. Despite their adaptations, diving mammals could be vulnerable to sustained and repetitive brain hypoxia. Future comparative pathological and neuroprotective investigations may prove of great value to Alzheimer's disease and other neurodegenerative diseases in humans.

***Key words*:**

neurodegenerative diseases, toothed whales, beaked whales, diving, hypoxia, neurofibrillary tangles, granulovacuolar degeneration, beta amyloid

BACKGROUND

Over 46 million people live with dementia worldwide and this number is estimated to attain global epidemic dimensions (131.5 million people) by 2050 [1]. Alzheimer's disease (AD) is a complex, multifactorial disease for which a number of genetic, environmental, and lifestyle risk factors have been identified [2]. Although there is a considerable body of knowledge on AD, this challenging disease of the new millennium is still considered a conundrum. Substantial research is in progress to understand the pathogenetic mechanisms of the disease and thus, to pursue a cure. Novel approaches on AD have identified a variety of neuroprotective phenomena . The pathologic hallmarks of AD are represented by the accumulation of beta-amyloid (A β) and neurofibrillary tangles (NFT) in neurons, neuroglia and neuroparenchyma, leading to neuronal and synaptic loss, and eventual brain atrophy [3].

A β comprises a 36 to 43 amino acids long peptide of the amyloid precursor protein (APP) hydrolysis. Indeed, the A β peptide is produced from APP through sequential cleavage of β - and γ - secretases while A β removal is dependent on the proteolysis and lysosome degradation system [3]. A β accumulates intracellularly and is the major component of the extracellular (neuroparenchymal) plaques found in AD brain tissue. Recent studies have suggested common mechanisms underlying neurodegenerative depositional diseases such as AD, Parkinson, or Huntington disease. Formation of amyloid-like protein aggregates unifies these superficially unrelated pathologies [4, 5].

NFT are composed of insoluble paired helical filaments of a highly phosphorylated form of the microtubule-associated protein τ (tau), and associated lipid. While typical NFT in central nervous system (CNS) have been reported in senior cats, increased phosphorylation of τ without typical NFT has been described in sheep and goat (*Cetartiodactyla*), cat, dogs, leopards, cheetah, bison, degu, wolverine, bear, American bison, [6]. In these cases, the τ changes tend to be sporadic and do not adopt 'full blown' features as seen in humans [6]. Again, evidence that A β pathology increases with age was reported in a large number of non-human primate species, New and Old World monkeys [6-8], and some great apes like the western lowland gorilla [9, 10].

On the contrary, NFT have rarely been described in non-human primate brain with the exception of a single aged chimpanzee [11] and in the gray mouse lemur [12]. Interestingly, some potential longevity in humans is much longer than great apes (40 to 55 years) [10] or even some species like the gray mouse lemur (*Microcebus murinus*) which is considered to be elderly over 5 years of age [12]. On the other side, the maximum recorded lifespan in the killer whale (*Orcinus orca*) is 78 years, in the short-finned pilot

whale is 63 years, and in the striped dolphin is 58 years [13, 14]. However, the choose of an ideal animal natural model is hampered by which animal appear to show species specific variations in the process of phosphorylation and cleavage of tau protein [15].

Positive labelling to τ was also observed in the cytoplasm of neurons of deep cortical areas of frontal, parietal and temporal lobe of one bottlenose dolphin (*Tursiops truncatus*) with no clear NFT [16]. On the other side, well-defined NFT were recently observed in the frontal and parietal cortex, thalamus, and cerebellum, of one bottlenose dolphin and three striped dolphins (*Stenella coeruleoalba*) [14].

Early-onset Alzheimer disease (EOAD), which presents in patients younger than 65 years, has frequently been described as having the same neuropathological hallmarks than late-onset Alzheimer disease (LOAD). However, higher burdens of neuritic plaques and NFT in frontal and parietal lobes have been found in EOAD than in LOAD patients [17]. On the other side, LOAD is more prevalent and generally has a more slowly progressing course as compared to EOAD [18].

While LOAD is consistently associated with aging [19], the plot thickens. In fact, vascular contributions to cognitive impairment and dementia (VCID; so called 'vascular dementia', 'vascular cognitive impairment' or 'vascular contributions to dementia') have been linked to AD [20]. Even though, the mechanisms by which hypoperfusion influences AD neuropathology remain unknown; decreased blood flow to the CNS in humans is classically associated with AD-related pathology. Some studies suggested that alterations in the CNS vasculature impair clearance of A β, and thereby accelerate the progression of AD [21].

As stated by Youssef *et al*., (2016), natural animal models of AD should recapitulate two major histopathological hallmarks: A β deposition and NFT formation. However, there are no natural animal models with comparable neurodegenerative findings. Moreover, most research into AD has been performed using transgenic rodents [6].

The order Cetacea comprises two extant suborders: Mysticeti (baleen whales) and Odontoceti (toothed whales). Cetacea along with manatees (Sirenia) are the only mammals that are fully adapted to life in water [22]. Toothed whales are hunters while baleen whales have plates along their upper jaws used to filter food from the water column. One of the most fascinating characteristics of the toothed whales is the exceptionally large size of the brain, both in absolute and in relative terms, and the extremely dense folding of the neocortex [23]. Cetaceans are homeotherms and long-lived top predators, which are at high risk of bioaccumulation and biomagnification of a variety of chemical pollutants [24].

Recent studies have drawn some novel attention to neurodegenerative diseases (NDD) in cetaceans and it has been suggested that dolphins might be one of the very few potential natural models of AD [14-16, 25-29].

Among toothed whales, the beaked whales (BW; family *Ziphiidae*) are particularly cryptic and comprise more than 22 different species [30]. BW routinely dive deeper than 1,000 m for about one hour in order to hunt deep-water squid and fish [30]. They surface with a substantial oxygen debt that is paid off in a prolonged surface time that includes a series of shallow non-foraging dives [30, 31].

In the last years, BW received public attention after a series of mass-strandings caused by mid-frequency naval sonars [32-34]. Decompression-like sickness (DCS) in the stranded animals was linked to naval mid-frequency sonar use.

A Cuvier's beaked whale (*Ziphius cavirostris*) broke the diving record at 3,000 m-depth lasted for two hours [30]. This is by far the deepest dive recorded for any air-breathing endotherm animal.

Diving mammals are regularly exposed to hypoxic conditions during breath-hold diving and a large body of literature has addressed how they maintain their body functions [22, 35-37]. Diving mammals rely on a large blood volume rich in hemoglobin and in skeletal muscles with high concentrations of myoglobin, resulting in an enhanced capacity for tissue oxygen storage [37]. Furthermore, molecular adaptations have also been demonstrated in the diving brain. Williams *et al.* (2008) described that neural globin proteins, neuroglobin and cytoglobin, facilitate the movement of oxygen from blood to neural tissues [38]. The concentration of these neural globins is inversely related to maximum dive duration in marine mammals: deep divers preferentially rely on circulating globins in the brain while faster swimming coastal species use enhanced neural globin proteins stores [38]. Neuroglobin is involved in conferring hypoxia tolerance in the diving brain [39], scavenging reactive oxygen and nitrogen groups and so defending against cellular damage [40]. Cetaceans evolved a strategy by maintaining remarkably high levels of neuroglobin mRNA in their brains [39]. Induction of resident neural globins, particularly neuroglobin, has been associated with neuronal survival following cerebrovascular accidents such as stroke [41]. Hence, during evolution, cetaceans had then increased their tolerance to hypoxia and decreased metabolism during dives.

Hypoxia has been pointed out as a risk factor that may accelerate AD pathogenesis by altering APP processing. Moreover, repeated hypoxia increases amyloid generation and neuritic plaques formation and also activates macroautophagy [42]. Also, a gradual decline of oxygen and glucose supply

to the brain during aging or hypoxic conditions has been demonstrated as a contributing factor to hypometabolism [19]. In AD patients, the brain regions with hypometabolism can trigger overexpression of APP and decrease the clearance of Aβ. Aβ and hypoxia can evoke inflammation, oxidative stress and eventual neuronal cell death [19].

On the other side, beneficial effects of the hyperbaric oxygen therapy (HBOT) have been described, holding great potential for the treatment of AD [43]. HBOT comprises the medical administration of 100% oxygen at conditions greater than 1 atmosphere absolute. The therapy seems to reduce hypoxia, amyloid burden, and τ phosphorylation and seems to meliorate symptoms experienced by patients with AD [43], but the results should be interpreted carefully due to its oxygen toxicity (heightened oxidative stress) [44].

RESULTS

We detected positive immunolabeling of varying intensity and extent for Aβ, NFT or both in 6 out of 9 (67%) animals (table 1). The immunohistochemical findings in these six animals are summarized as follows.

Case 1 (*Stenella frontalis,* elderly, Fig. 1) presented A β plaques in the frontopolar cortex. Also, there was strong and diffuse nuclear A β labeling throughout the five neuronal layers of the frontopolar cortex involving the 3 types of neurons: pyramidal, granular, and Von Economo neurons, a type of large spindle-shaped neurons in layer V. Intranuclear staining was also detected in Purkinje neurons of the anterior and posterior cerebellum. The frontopolar cortex had scattered cortical neurons with diffuse cytoplasmic NFT labeling.

Case 2 (*Ziphius cavirostris*, subadult) had mild to moderate nuclear staining to A β in the neurons of the frontal neocortex and in the cerebellar Purkinje neurons. A β plaques were not observed.

Case 5 (*Mesoplodon densirostris*, adult, Fig. 2) had widespread granulovacuolar cytoplasmic labeling to NFT in Purkinje neurons. This animal also had cerebral nasitremiasis confined to the forebrain (unpublished data).

Case 6 (*Stenella frontalis*, adult) had mild, multifocal granular to vacuolar, cytoplasmic labeling to NFT in scattered Purkinje neurons.

Case 7 (*Stenella frontalis*, adult) had scattered cortical neurons with diffuse cytoplasmic NFT labeling. No granular or vacuolar labeling was detected.

Case 8 (*Mesoplodon densirostris*, subadult) had scattered neurons with granular to vacuolar cytoplasmic labeling for NFT in Purkinje neurons.

DISCUSSION

Herein, we present immunohistochemical evidence of CNS positive labeling for Aβ and NFT in 6/9 (67%) animals, representing three odontocete species. Aβ immunopositivity involved cerebral and cerebellar neurons and A β plaques, and NFT immunopositivity was confined to cerebellar Purkinje neurons with granulovacuolar degeneration as well as in the cytoplasm of scattered frontopolar neurons as diffuse staining. These findings were more extensive in deep and long-lasting diver species (one *Z. cavirostris* and two *M. densirostris*); however, they were also found in one elderly and two adult, shallow-diver species, namely *S. frontalis*. Two of three BW positive for Aβ and NFT were subadults and the other was adult. Based on these results, we present a novel hypothesis that considers hypoxia as one of the most important risk factors that could contribute to NDD in cetaceans, with special attention to BW (Fig. 3), alongside to other proposed risk factors/causes proposed by other authors [14, 25, 27, 28, 33].

Intranuclear neuronal Aβ-labeling: what does it mean?

Intranuclear neuronal Aβ-labeling is barely described in the literature. The cell nucleus is a major target of amyloid-like protein fibrillation in a variety of disorders that are characterized by widespread aggregation of proteins with instable homopolymeric amino acid repeats, ubiquitin, and other proteinaceous components [4].

It has been suggested that Aβ is deposited in the vicinity of DNA in the nuclear region of AD cells [45]. Intranuclear neuronal Aβ-labeling was observed in two cases in this study: an elderly *S. frontalis* and a subadult *Z. cavirostris*. The later was one of the 14 BW stranded in close temporal and geographic association with an international naval exercise (Neo-Tapon 2002) held on September 24th, 2002 [46, 47]. In this animal, we propose intranuclear neuronal Aβ expression having a neuroprotective role to hypoxia.

In fact, previous studies have demonstrated that intracellular Aβ42 accumulation is an early event in neuronal dysfunction. Furthermore, preventing intraneuronal Aβ42 aggregation may be an important therapeutic direction for the treatment of AD [48]. Even though the mechanism is still unknown and there is no confirmation to date on Aβ binding to DNA, the nuclear localization of Aβ might play a role in bringing about changes in DNA topology, modulating both helicity and superhelicity in super coiled DNA [45]. In addition, AD-vulnerable brain regions specifically accumulate γ-cleaved Aβ42, suggesting that intraneuronal Aβ42 immunoreactivity appears to precede both NFT and Aβ plaque deposition [48].

Although the role of nuclear amyloid is still unknown, the Aβ intranuclear expression provides new insights regarding cerebral safeguards for hypoxic-ischemic brain injury from accidents or disease. While it was initially thought that nuclear amyloid is responsible for neural cell death, time-resolved experiments that correlate nuclear amyloid and neurodegeneration on the single cell level also suggest a cell protective role [4].

Granulovacuolar degeneration for selectively vulnerable neurons

Neuronal granulovacuolar degeneration (GVD) is one of the histopathological hallmarks of AD and is defined as electron-dense granules (0.5 to 1.5 mm in diameter) within double membrane-bound cytoplasmic vacuoles, mainly in the hippocampal pyramidal neurons [49]. The granules are composed of several components including neurofilament proteins, ubiquitin, phosphorylated τ, and other microtubule-associated proteins [50]. To the best of the authors' knowledge, GVD has not been observed in the very few studies performed in aged rats, wolverines, and dogs [51-53]. Even though, in the gray mouse lemur, τ proteins was observed as clumps of thick granules located close to the membrane of the neuronal perikarya and the dendrites [12].

GVD was observed in the Purkinje neurons of the anterior and posterior cerebellum of two BW and one *Stenella frontalis*. GVD is not pathognomonic of AD and can be observed in other NDD and in brains of non-demented aged people [54]. GVD inclusions and hyperphosphorylated forms of the microtubule-associated protein τ (which comprise the neurofibrillary changes) are present in the same neurons, and neurons with GVD frequently occur together with those containing NFT [54]. Several antibodies to τ inconsistently label GVD indicating that the pattern of GVD immunoreactivity shows a phosphorylation state (hyperphosphorylation) of the granules that is similar to that of NFT [54]. GVD inclusions are autophagic vacuoles that may sequester proteins. Their role is not yet resolved and may be interpreted as either a cellular defense mechanism or an indicator of impaired cellular functioning [54]. Regardless of whether GVD is a cellular defense mechanism or not, this finding has never been documented in any marine mammal species [55]. We did not evaluate the hippocampal formation in this preliminary study; future studies should address potential key roles of this neuroanatomical locale, so tiny in cetaceans, in NDD in marine mammals [56]. Certain structures of the hippocampus are vulnerable as are the Purkinje neurons of the cerebellum [57]. This would provide further support to "selectively vulnerable" regions of the brain under hypoxic conditions. Lastly, the cerebellum has to be firmly considered as a key protagonist in the pathophysiology and manifestations of AD and other NDD [58,

59], including marine mammal species. Interestingly, Purkinje cell loss and cerebellar astrocytosis in familial Alzheimer's disease (FAD) are greater than in LOAD, indicating that the cerebellum is more affected in FAD than in LOAD [60].

Aβ and NFT: no cross-reactivity of shared epitopes

The specific patterns of intraneuronal Aβ and granular (and in a few cases, diffuse) NFT labeling would exclude the possibility of cross-reactivity of shared epitopes. Additionally, the specificity of the A β uptake was attested to by comparison with Congo red staining; there was complete correlation between the Congo red staining visualized in the neuronal nuclei and the reference compound A β. Moreover, the Congo red staining validated the immunopositivity not only in the elderly plaques but also in the nuclei of the neurons and confirmed the A β uptake.

CONCLUSIONS

Herein, we present immunohistopathological findings classic of Alzheimer's disease and other NDD in humans, *i.e.*, A β and NFT labeling, in different CNS locales of odontocete cetaceans. These findings could be linked to hypoxic phenomena, as they were more extensive in the brains of deep diver cetacean species, specifically beaked whales, and not only in aged individuals. Therefore, a novel hypothesis linking hypoxia and NDD microscopic hallmarks in cetaceans is proposed.

This study presents the first description of A β and NFT in the brain of BW, adding also to the few descriptions of GVD in the brain of non-experimental animals, being specifically the first report of GVD in the cerebellum. This research further confirms the rarely reported intranuclear expression of A β. Despite their adaptations, diving mammals could be vulnerable to sustained and repetitive CNS hypoxia. Future investigations should also address potential neuroprotective adaptations in these species. Drawing a specific fingerprint-like pattern of the behavior of neuronal and non-neuronal components of the brain [33] could help to better understand the pathogenesis of some NDD. Further research is needed in cetaceans, with special attention to deep diver species and BW; comparative pathological and neuroprotective investigations may prove of great value to AD and other NDD in humans.

In summary, these results found evidence of both amyloid deposits and tau pathology in stranded toothed whales, making them one of very few naturally occurring models of AD as well as other NDD.

Finally, NDD should be reclassified according to the cell biology pathways affected rather than only by the mere presence of plaques and tangles as in AD. Nowadays, there is evidence that some NDD

have both clinical and genetic overlaps and that most of the patients with AD, if they survive long enough, develop a movement disorder of the Parkinson's type. Even if we detected Aβ deposition and NFT by immunohistochemistry as GVD, we do not state these animals have AD. We strive to provide histopathological and immunohistochemical commonalities with human NDD, including AD and surmise some factors, particularly hypoxia, may play a role in their neuropathogenesis.

METHODS

Animals, brain processing methodology and histopathological analysis

One of the main challenges of working with cetaceans' brains is to establish a valid methodology for an optimal manipulation and fixation of such specimens, for neuroanatomical and neuropathological studies. Such difficulties are related to (1) their brain size, (2) the logistic difficulties and laboratorial complexity to achieve a large sample size from certain elusive species, such as the BW – here the Cuvier's beaked whale (*Ziphius cavirostris*) and the Blainville's beaked whale (*Mesoplodon densirostris*), and (3) the difficulties to obtain 'extremely fresh' brain samples from stranded individuals.

The brain including cerebrum, cerebellum, brainstem and spinal cord of eight animals representing six different odontocete species that stranded and died along the coastline of the Canary Islands (28°17'29"N, 16°37'44"W; Spain), were included in this study. On one side, five deep divers animals were included in the study: one Cuvier's beaked whale, two Blainville's beaked whales, one short-finned pilot whale (*Globicephala macrorhynchus*), one Risso's dolphin (*Grampus griseu*s). Additionally, 4 shallow divers were also added: three Atlantic spotted dolphins (*Stenella frontalis*), and finally one captive neonatal bottlenose dolphin, which died of natural cause, was examined as control.

The required permission for the management of stranded cetaceans was issued by the environmental department of the Canary Islands' Government and the Spanish Ministry of Environment. Neither experiments were performed on live animals, nor these animals were sacrificed for the purposes of the study.

The animals were of different age categories: elderly (n=1), adult (n=5), subadult (n=2), newborn (n=1), based on total body length, gross and microscopic gonadal appearance [61], and systemic gross and microscopic features, e.g., pronounced tooth wear, neuronal lipofuscinosis, greater amount of neuromelanin in the *locus coeruleus* [26] or *substantia nigra*, intraneuronal polyglucosan bodies, leptomeningeal fibrosis, and choroid plexus hyalinosis.

Brains were removed from the neurocranium carefully and promptly immersion-fixed at necropsy in 4% formaldehyde solution in phosphate-buffered saline (PBS; pH 7.4) and processed for neuroanatomical and neuropathological analysis as described by Sacchini *et al.* [26]. After rinsing in PBS, samples were cryoprotected in 30% sucrose solution in PBS (pH 7.4) at 4°C, to avoid freezing artifacts. The samples were immersed in a mixture of PBS-sucrose and Optimum Cutting Temperature formulation (OCT) (1:1) overnight. The day after, the samples were included in a mold using the same mixture, were rapidly frozen and 50-60 μm-thick serial sections were obtained employing a cryostat (Leica CM1950, Nussloch, Germany). Sections were stored in PBS (pH 7.4) solution with sodium azide (0.01%).

Additionally, for microscopic analysis, formalin-fixed, paraffin-embedded (FFPE) tissue sections were processed as routine, cut at 5 μm-thick and stained with hematoxylin and eosin.

Immunoperoxidase staining

The immunoperoxidase staining procedure was carried out on free-floating sections as described in Sacchini *et al*. [26]. Commercially available, human-oriented primary antibodies (pAbs) used were a monoclonal anti-Aβ (1:100, clone H31L21, Invitrogen, Carlsbad, CA), and a polyclonal anti-NFT (1:100, Invitrogen) incubated 48 hours at 4°C. To block non-specific binding, sections were incubated in a solution containing normal goat serum (S-1000, Vector Laboratories, Burlingame, CA), and 0.5% Triton X-100 (Merck, Darmstadt, Germany). The sections were then incubated for 45 minutes with a biotinylated goat anti-rabbit antibody (BA-1000, Vector Laboratories) diluted 1:200 in a solution containing 1% normal goat serum in PBS. No block for endogenous biotin was used. The immunoreactions were visualized either by 3,3'-diaminobenzidine (DAB) peroxidase kit (SK-4100, Vector Laboratories, city, country) followed by counterstaining with thionine, or by 3-amino-9-ethylcarbazole (AEC) peroxidase kit (Vector Laboratories, SK-4200) without counterstaining.

Bennhold's Congo Red stain

Congo red is the most popular dye used as a probe for diagnosing amyloidosis also in AD brains. The Bennhold's Congo red stain is commonly used for the detection of amyloid on FFPE and frozen tissue sections [62]. The amyloid deposits are stained red and the nuclei are stained blue. FFPE samples were deparaffinized with xylene and rehydrated in graded ethanol. Sections were stained with Congo red solution for 1 hour, rinsed in distilled water, differentiated quickly in alkaline alcohol solution, and

counterstained with Mayer's hematoxylin. The positive control was a kidney from a dog with severe renal amyloidosis.

Abbreviations

AD Alzheimer's disease; AEC 3-amino-9-ethylcarbazole; APP amyloid precursor protein; Aβ β-amyloid; BW beaked whales; CNS central nervous system; DAB 3,3'-diaminobenzidine; DCS Decompression-like sickness; EOAD Early-onset Alzheimer disease; FAD familial Alzheimer's disease; FFPE formalin-fixed paraffin-embedded; GVD granulovacuolar degeneration; HBOT hyperbaric oxygen therapy; LOAD late-onset Alzheimer disease; NDD neurodegenerative diseases; NFT neurofibrillary tangle; OCT Optimum Cutting Temperature; pAbs human-oriented primary antibodies; PBS phosphate-buffered saline; τ tau

Ethics approval and consent to participate

The required permission for the management of stranded cetaceans anywhere within the Canarian archipelago was issued by the environmental department of the Canary Islands' Government. No experiments were performed on live animals because our work is based on dead stranded cetaceans.

Consent for publication

Not applicable.

Availability of data and materials:

All data for this study are included in the manuscript and the supplementary files. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions:

SS and AF contributed conception and design of the study. SS, JD, AE, YP, YB, ES, MA, PH, and AF contributed to organization of the databases and/or collected samples for histopathological and immunohistochemical analysis. SS, YP, and ES contributed to laboratorial resources. SS wrote the first

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Authors Informations

[1]Division of Histology and Animal Pathology, Institute for Animal Health and Food Security (IUSA), Veterinary School, Universidad de Las Palmas de Gran Canaria, 35416 Arucas, Gran Canaria, Canary Islands, Spain. [2]Laboratory of Wildlife Comparative Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, SP, Brazil. [3]Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL), College Station, TX, USA.

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Figure 1: Case 1 (*Stenella frontalis*).

Strong and diffuse nuclear staining to Aβ in the cortical neurons. Aβ free-floating immunolabelling; DAB not counterstained with thionine (A) Bennhold's Congo Red stain (B). Scale bar = 20 µm. Aβ-positive aggregate in the frontopolar cortex (C). There are multiple irregular, slightly well-demarcated dense Aβ-positive extracellular/neuroparenchymal aggregates (D, asterisks) in the gray matter and subcortical white matter. Aβ free-floating immunolabelling; DAB counterstained with thionine.

Figure 2: Case 5 (*Mesoplodon densirostris*).

NFT-granulovacuolar labeling in Purkinje neurons (A-C). Purkinje cells have focal to diffuse granular cytoplasmic NFT-positive labeling (A and C). Free-floating immunolabelling; DAB counterstained with thionine (A and C). AEC not counterstained (B).

Figure 3: Neurodegenerative diseases in diving marine mammals (here represented a beaked whale) may result from the interactive effects of multiple risk factors among which hypoxia could be one of the most important.

Supplementary material

Table 1: Stranding and biologic data for nine odontocetes included in this study and immunohistochemical results for β-amyloid and neurofibrillary tangle immunomarkers in cerebral cortex and cerebellum. Semiquantitative analysis of intensity and extent: –, no; +,mild; ++, moderate; +++, high. * neonatal dead animal used as control. NA, not applicable.

Figures

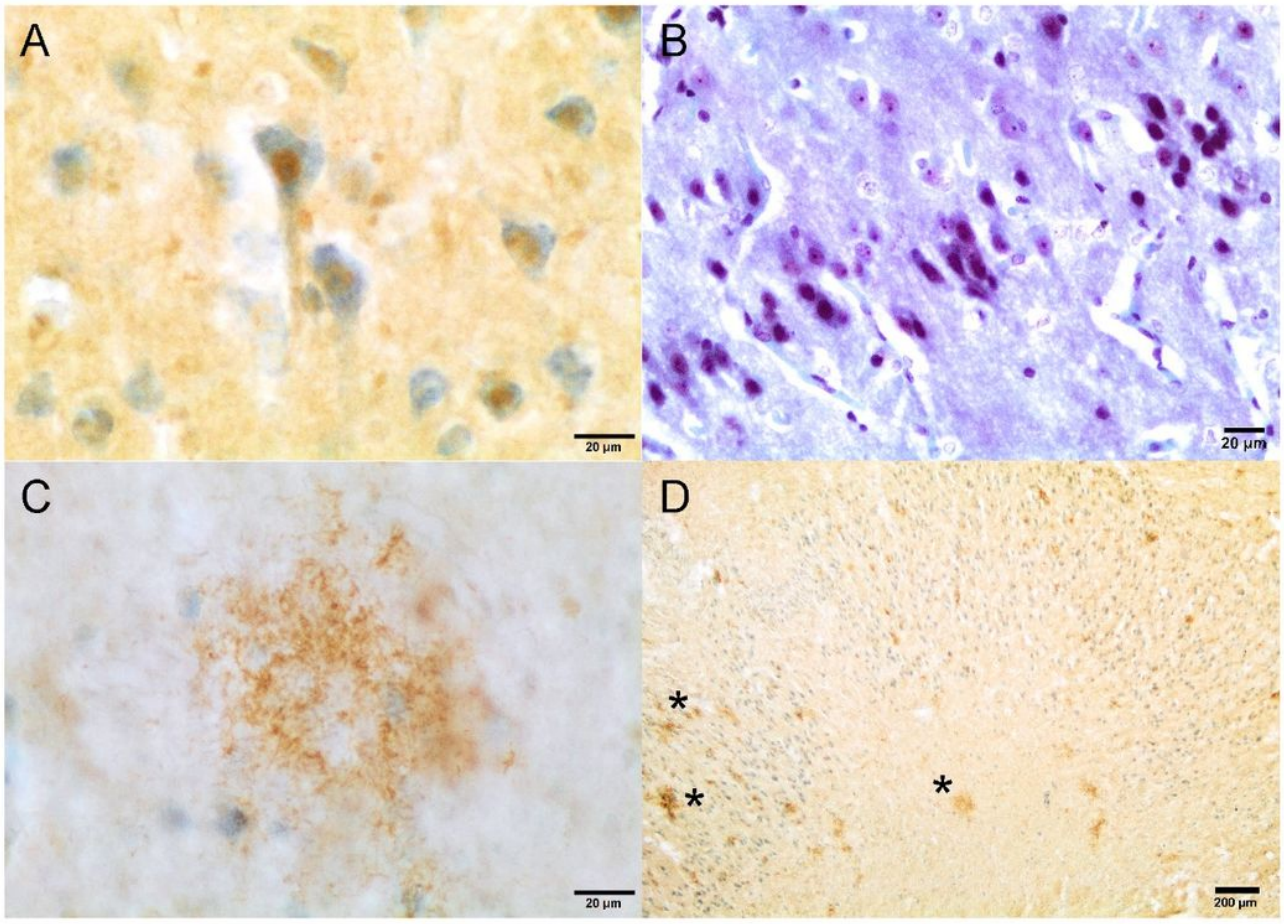

Figure 1

Case 1 (Stenella frontalis). Strong and diffuse nuclear staining to Aβ in the cortical neurons. Aβ free-floating immunolabelling; DAB 22 not counterstained with thionine (A) Bennhold's Congo Red stain (B). Scale bar = 20 µm. Aβ-positive aggregate in the frontopolar cortex (C). There are multiple irregular, slightly well-demarcated dense Aβ- 24 positive extracellular/neuroparenchymal aggregates (D, asterisks) in the gray matter and subcortical white matter. Aβ free-floating immunolabelling; DAB counterstained with thionine.

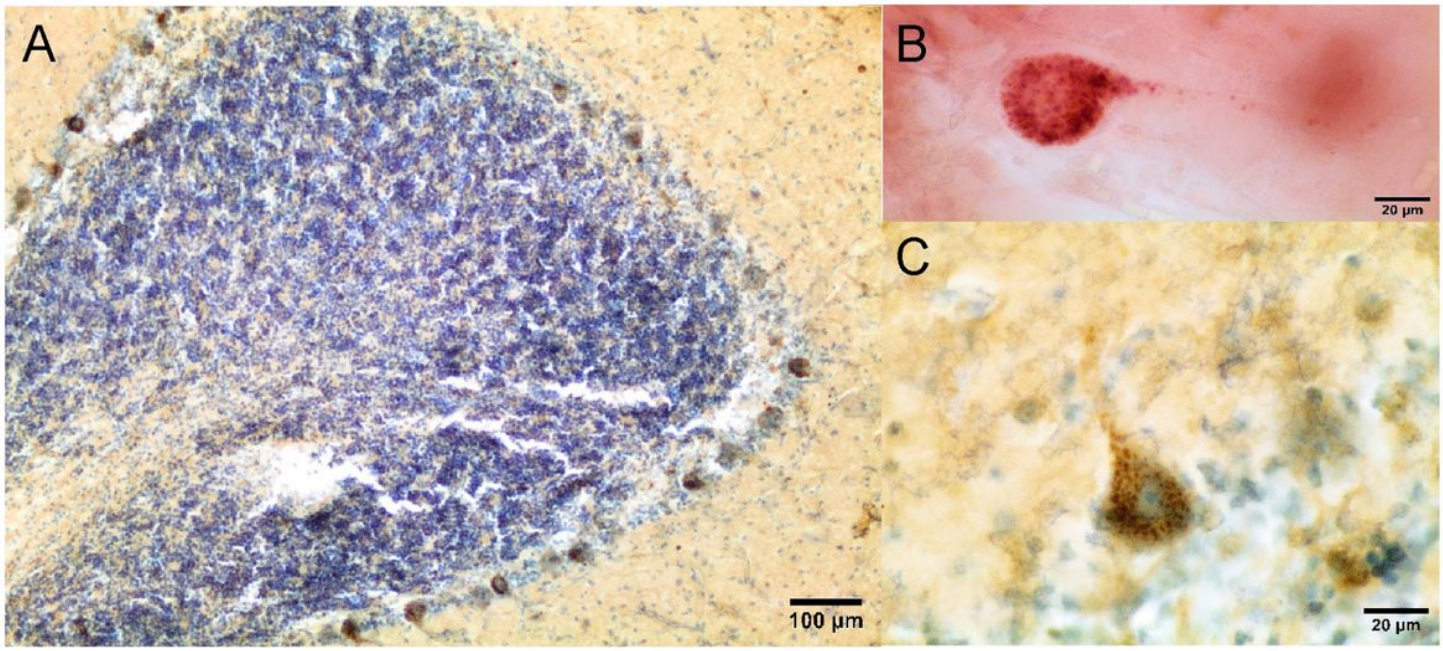

Figure 2

Case 5 (Mesoplodon densirostris). NFT-granulovacuolar labeling in Purkinje neurons (A-C). Purkinje cells have focal to diffuse granular cytoplasmic NFT-positive labeling (A and C). Free-floating immunolabelling; DAB counterstained with thionine (A and C). AEC not counterstained (B).

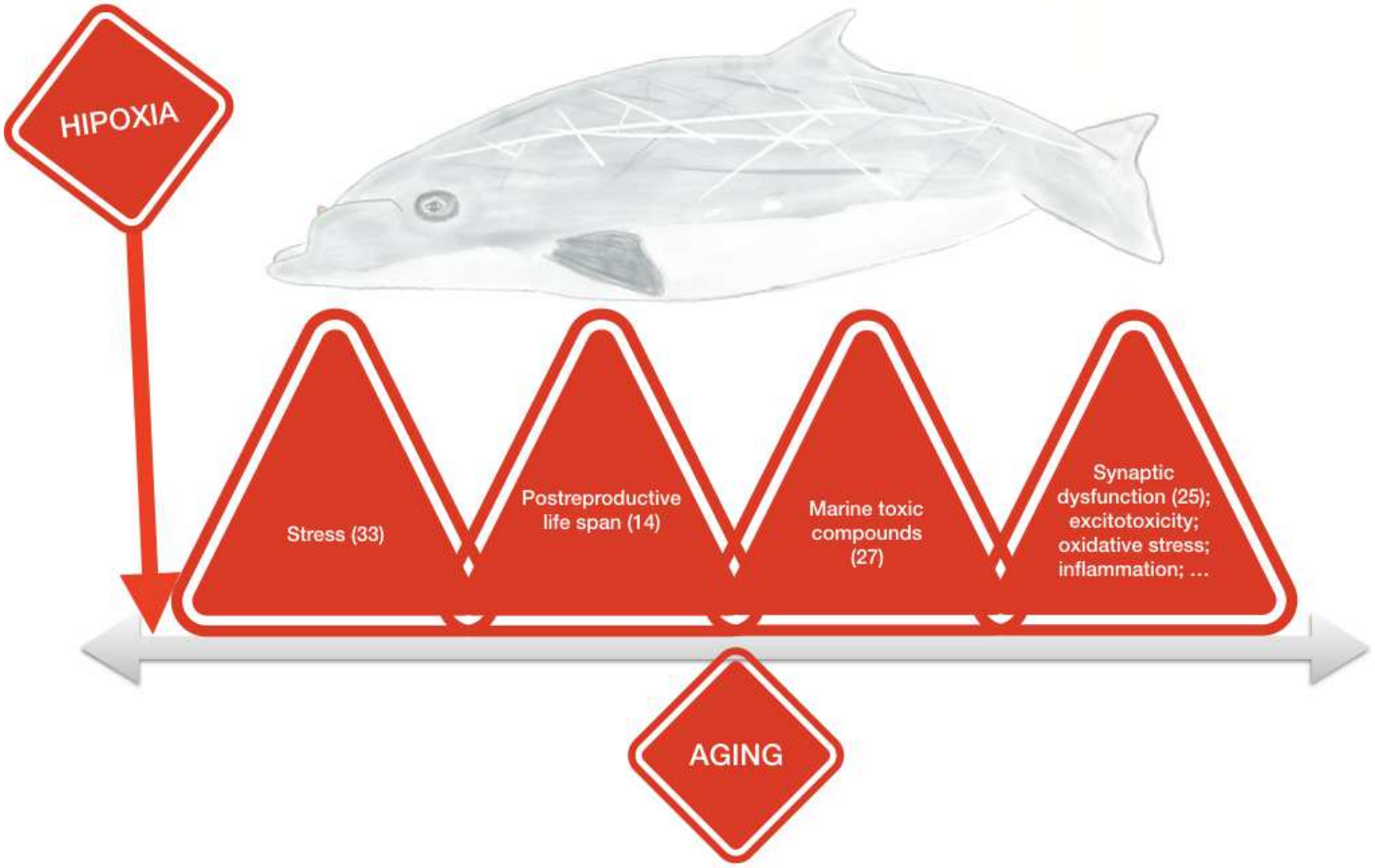

Figure 3

Neurodegenerative diseases in diving marine mammals (here represented a beaked whale) may result from the interactive effects of multiple risk factors among which hypoxia could be one of the most important.

Supplementary Files

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