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The nervous system of *Delphinidae*: neurochemical studies on different central and peripheral regions

Presentata da: Anna Maria Rambaldi

Coordinatore Dottorato	Relatore
Chiar.mo Prof. Arcangelo Gentile	*Chiar.mo Prof. Cristiano Bombardi*

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INDEX

ABSTRACT

During the evolutionary path, Cetaceans experienced a return to waters and hence had to adapt many of their anatomical and physiological features to this new life. Many organs and systems present several modifications and specialisations, which make these mammals different from their mainland ancestors. The nervous system either displays peculiar features like an extremely large brain, in terms of both absolute and relative mass, a very high level of gyrification, a minimization, or in some cases a complete lack, of olfactory structures, and a poorly developed *corpus callosum*. Despite a strong interest in studying these peculiarities, mainly because of its sociocognitive implications, to date, for many areas of the cetaceans nervous system, a detailed anatomical, histological and neurochemical description is lacking. This is explained by a variety of limitations, including difficult availability of samples, preservation state of the carcasses, etc… The aim of the present research is to investigate the neurochemistry of different central and peripheral regions of the *Delphinidae* nervous system, including anatomical and histological features.

Firstly, we developed two anatomical studies on the central nervous system of healthy bottlenose dolphins:

1- "Distribution of Calretinin Immunoreactivity in the Lateral Nucleus of the bottlenose dolphin (*Tursiops truncatus*) Amygdala".

2- "Calcitonin Gene-Related Peptide (CGRP) expression in the spinal cord and spinal ganglia of the bottlenose dolphin (*Tursiops truncatus*)".

Subsequently, we focused on the peripheral nervous system, in particular on the enteric nervous system of healthy dolphins:

3- "Nitrergic and substance P immunoreactive neurons in the enteric nervous system of the bottlenose dolphin (*Tursiops truncatus*) intestine".

Finally, we developed a research project on the expression of three neural markers in different brain regions of striped dolphins under pathological and no pathological conditions:

4- “Preliminary study on the expression of calcium binding proteins and neuronal nitric oxide synthase (nNOS) in the cortex and cerebellum of striped dolphins (*Stenella Coeruleoalba*) affected by morbillivirus”.

Distribution of Calretinin Immunoreactivity in the Lateral Nucleus of the Bottlenose Dolphin (*Tursiops truncatus*) Amygdala

The amgdaloid complex consists of different nuclei, each with unique cytoarchitectonic, chemoarchitectonic and connectional features. The majority of inputs coming from cortical and subcortical areas enters the amygdala via the lateral nucleus, which makes it the main receiving structure of the complex. The activity of its neurons is coordinated and modulated by a variety of inhibitory GABAergic interneurons, which can be classified according to expression of different calcium-binding proteins as well as by morphological parameters. The present research, based on the analysis of the amygdala of three bottlenose dolphins, provides the first description of the topography, cytoarchitecture and distribution of calretinin immunoreactivity of the lateral nucleus. Our observations confirmed the general topography of the mammalian amygdala and in particular of the lateral nucleus of the bottlenose dolphin. Notably, we identified six subdivision of the nucleus, more than what reported until now in the rat, monkey and human lateral nucleus. This could reveal an outstanding capability of integration and elaboration of external stimuli. In addition, we observed a strong presence of CR-immunoreactive (-IR) neurons and fibres. CR-IR neurons were mainly non-pyramidal inhibitory neurons; in particular, the 80% of IR-cells were represented by large and small polygonal neurons. Since in the lateral nucleus of the human amygdala, CR-IR neurons form inhibitory synapses on calbindin-D28k-IR inhibitory interneurons, which—make inhibitory synapses on the pyramidal cells, we can suppose/hypotize that in the bottlenose dolphin CR-IR interneurons may be involved in the synchronization of cells activity, thus playing an important role in the control of information flow in the lateral amygdalar nucleus.

Calcitonin Gene-Related Peptide (CGRP) expression in the spinal cord and spinal ganglia of the bottlenose dolphin (*Tursiops truncatus*).

The localization and function of neurotransmitters in the spinal cord and spinal ganglia has been investigated mostly in laboratory animals. Data on large mammals are scant and the neurochemistry of spinal cord and spinal ganglia of Cetaceans has been studied only in part. Sensory neurons, especially nociceptive sub-populations, are generally grouped in two classes, peptidergic and non-peptidergic. Calcitonin gene-related peptide (CGRP) represents, together with Substance P (SP), one of the most used markers of the peptidergic class. On the other hand, non-peptidergic neurons are usually identified, at least in mice, as isolectin B4 (IB4) binding cells. In the present research, the expression of CGRP, was deeply investigated in the caudal spinal cord and corresponding spinal ganglia of the bottlenose dolphin (*Tursiops truncatus*). Distribution, mean perikaryal area and density of neurons were measured in cresyl violet- and immunoperoxidase-stained sections. The expression of SP and IB4 was also investigated by immunofluorescence technique. The distribution of CGRP immunoreactivity in the dolphin spinal cord and corresponding spinal ganglia resulted very similar to what described in other mammals. In lamina IX CGRP-IR cells account for the 81 % of total motor neurons while in spinal ganglia, CGRP-IR neurons represent the 40% of neuronal population. Differently from rodents, in the spinal cord of the bottlenose dolphin, CGRP, SP and IB4-IR fibers mostly share the same central terminations in the dorsal horn, mainly localized in the first two laminae. Also in the spinal ganglia, IB4 was co-localized with CGRP and SP, allowing us to conclude that IB4 is not indicated as a marker of non-peptidergic neurons in the bottlenose dolphin. As reported for other species, the large presence of CGRP in sensory and motor system of the bottlenose dolphin is suggestive of a heterogeneous functional picture for this peptide.

Nitrergic and substance P immunoreactive neurons in the enteric nervous system of the bottlenose dolphin (*Tursiops truncatus*) intestine

Compared with other mammals, the digestive system of cetaceans presents some remarkable anatomical and physiological differences. However, in these animals the neurochemical features of the enteric nervous system (ENS) have been described only in part. The present study provides a description of the nitrergic and selected peptidergic systems in the myenteric plexus (MP) and submucosal plexus (SMP) of the intestine of the bottlenose dolphin (*Tursiops truncatus*). The distribution and morphology of nNOS- and SP- IR neurons were immunohistochemically studied in formalin-fixed specimens from healthy animals, and data were compared with what described in other mammals (human and non-human). Although morphological features of nNOS- and SP-IR neurons were similar to what reported in other mammals, we found some remarkable differences in the number of bottlenose dolphin enteric neurons; in fact in this species, we detected a lower number of nNOS-IR neurons in the SMP and a higher number of MP SP-IR neurons compared to other mammals. To the best of Authors' knowledge, this study represents the first description and quantification of nNOS-IR neurons and the first quantification of SP-IR neurons in the intestine of a cetacean species. As nNOS and SP are important mediators of intestinal functions and nitrergic population represents an important target of many neuroenteropathies, data obtained in the intestine of healthy dolphins represent a necessary basis to better investigate and understand possible functional differences and motor intestinal dysfunctions/alteration in these peculiar mammals.

Preliminary study on the expression of calcium binding proteins and neuronal nitric oxide synthase (nNOS) in the cortex and cerebellum of striped dolphins (*Stenella Coeruleoalba*) affected by morbillivirus

Non suppurative meningoencephalitis is a common form of inflammation reported in many species of marine mammals. In cetaceans, it recognizes different causative agents like morbillivirus, herpesvirus, Toxoplasma gondii e Brucella spp.. Some calcium-binding proteins, like calretinin

(CR) and calbindin D-28k (CB-D28k), play an important role in neuroprotection, buffering the excess of free intracellular calcium ions. The distribution of these markers under physiological conditions has been already described in some brain regions of different cetacean species. Neuronal nitric oxide synthase (nNOS) is an enzyme that catalyses nitric oxide (NO) production in the nervous system. NO, whose synthesis is Ca^{2+}-dependent, is a neurotransmitter with neuroprotective functions in physiological amount but potentially neurotoxic when overexpressed. Four striped dolphins (*Stenella coeruleoalba*) with confirmed morbilliviral meningoencephalitis and three not-infected controls were included in the study. Immunohistochemistry was performed on formalin fixed, paraffin embedded tissues (i.e. cortex and cerebellum). In order to evaluate the percentage of immunoreactive area, frames for each slide were recorded and analysed with the image analysis software ImageJ and statistical analysis was performed. The distribution of CB-D28k and CR confirms what previously described in other toothed whales species. As far as we are aware, here we report the first description of nNOS-immunoreactivity in the neocortex and cerebellum of a cetacean species. Although both slide observation and ImageJ analysis prove a decreased expression in affected individuals, mainly involving the cortex, statistical analysis reveals a significant difference only for the CB-D28k expression in the cortex. Despite many efforts, technical/practical limitations, as non-homogeneous sampling, preservation state of the carcasses and different forms and stages of the meningoencephalitis, make this research, albeit original, a preliminary step, which might be necessary enhanced, in order to clarify a potentially plasticity and involvement of these markers in morbilliviral meningoencephalitis.

This collection of studies focuses on different neurochemical aspects of some central and peripheral regions of *Delphinidae* nervous system, under physiological and pathological conditions. Although many of the characteristics reported here match what previously described in other

mammals, some differences have been observed. We tried to confer to such variations a physiological implication in the context of the evolutionary process that cetaceans sustained.

Several aspects that make these mammals so fascinating usually constitute an obstacle and a challenge for the research. Thus, many of their characteristics and morpho-physiological peculiarities have not yet been studied or fully understood. This collection of studies is a little contribution to the current knowledge on cetaceans' nervous system. We believe that anatomy and other basic sciences represent the necessary way that leads to a better knowledge and understanding of these peculiar animals, with a view to planning proper and effective conservation strategies.

INTRODUCTION

CHAPTER 1

Cetaceans and general adaptations to aquatic environment

Cetaceans are a group of widely distributed aquatic mammals, which currently includes 92 species (Perrin, 2017). They have been traditionally considered as an order, further divided in the suborders Odontocetes (toothed whales) and Mysticetes (baleen whales). More recently, molecular studies have demonstrated the close relationship between *Cetacea* and *Artiodactyla*, in particular between cetaceans and hippopotamids (Shimamura et al., 1997; Price et al., 2005). This led to the fusion of the two groups in a new order, named *Cetartiodactyla*. Differently from baleen whales, toothed whales are provided with teeth (as the name suggests), a specialized echolocation system, and are characterized by the presence of a single blowhole (Perrin et al., 2009). The suborder of toothed whales consists of four superfamilies, Physeteroidea, Ziphioidea, Platanistoidea and Delphinoidea. Within this latter group, three families can be distinguished, namely *Monodontidae* (narwhale and the Beluga), *Phocenidae* (porpoises) and *Delphinidae* (dolphins). *Delphinidae* consists of many different morphological and ecological types and represents the largest and most studied group. In particular, the bottlenose dolphin (*Tursiops truncatus*) is the most common and well-known member of its family, being the subject of myriads of studies in many fields covering, for example, anatomy, ecology, pathology and so on. The reason probably lies in its worldwide and often coastal distribution and in its curious nature. Moreover, the bottlenose dolphin is the cetacean species most frequently held in captivity and, besides ethical questions, this condition provides a remarkable and longlasting "accessibility", compared with wild animals.

More than 50 million years ago, cetaceans' terrestrial ancestors returned to an entirely aquatic lifestyle (Thewissen et al., 2007). This evolutionary path required many anatomical and physiological rearrangements that permitted cetaceans to perfectly adapt to marine and freshwater. For many aspects, aquatic environment means several challenges for animals who carry a lot of their terrestrial ancestors' baggage. Among fascinating modifications, we would like to mention the

transformation of the toothed whales upper respiratory tract in a biosonar (Cranford et al., 1996; Au et al., 2006; Berta et al., 2014), the reduction, or even lack, of the extremities in order to gain hydrodynamics (Reidenberg, 2007), the presence of a thick layer of subcutaneous adipose tissue (blubber) and countercurrent heat exchange systems to avoid heat losses, and all those anatomical and physiological adaptations to diving. Cetaceans can handle the increasing pressure during diving because of a jointed and collapsible thorax. Furthermore, as other diving mammals, the skeletal muscles of cetaceans are specifically designed to maintain a low profile of aerobic metabolism under the hypoxic conditions of diving, and store high amount of oxygen (Ridgway and Howard, 1979; Snyder, 1983; Noren and Williams, 2000; Noren et al., 2001; Kanatous et al., 2002; Wright and Davis, 2006) (Fig. 1.1).

TABLE I
Distribution and Quantity of Oxygen Stores, Maximum and Routine Diving Depths, and Durations for Some Marine Mammals

Species	*Body mass (kg)*	*Total store (ml/kg)*	*Lung*	*Blood (%)*	*Muscle*	*Routine depth (m)*	*Maximum depth (m)*	*Routine duration (min)*	*Maximum duration (min)*
Human	70	20	24	57	15	5	214	0.25	6
Weddell seal	400	87	5	66	29	200	741	15	93
Elephant seal	400	97	4	71	25	500	1,653	25	120
California sea lion	100	40	21	45	34	40	275	2.5	10
Bottlenose dolphin	200	36	34	27	39		535		
Cuvier's beaked whale	3,000					1,070	1,888	58	85
Sperm whale	10,000	77	10	58	34	500	2,035	40	73

Note: There is an extensive list of diving capabilities of many species of diving animals at: http://polaris.nipr.ac.jp/~penguin/penguiness/index.html

Fig. 1.1 Characteristics of some long/deep and short/shallow diving marine mammals compared with human. From Encyclopaedia of Marine Mammals, 2nd edition, Perrin W., Würsig B., Thewissen J.G.M, Academic Press, 2008.

Another noteworthy morphological feature is the great development of *retia mirabilia*, literally "wonderful nets", in many districts of the body. Described for the first time by Tyson (1680) in the

harbor porpoise (*Phocoena phocoena*), *retia mirabilia* are specialized vascular structures, which consist of extensive plexuses of anastomosing arteries and/or veins. The most developed *retia* are those found in the thoracic cavity, head and neck, although tiny *retia* can be also observed in other districts (Berta et al., 2006; Cozzi et al., 2017) (Fig. 1.2). Although the role of these vascular nets has not yet been fully understood, they seem to be crucial for diving, when probably part of the blood is diverted here. Hypotheses about the role of the *retia* include modulation of hemodynamics, storage of oxygen and thermoregulation. Furthermore, it is possible that, during diving, *retia* prevent the formation of nitrogen bubbles and the displacement of abdominal organs when the lungs collapse (Hui, 1975; Reidenberg, 2007; Blix et al., 2013; Cozzi et al., 2017).

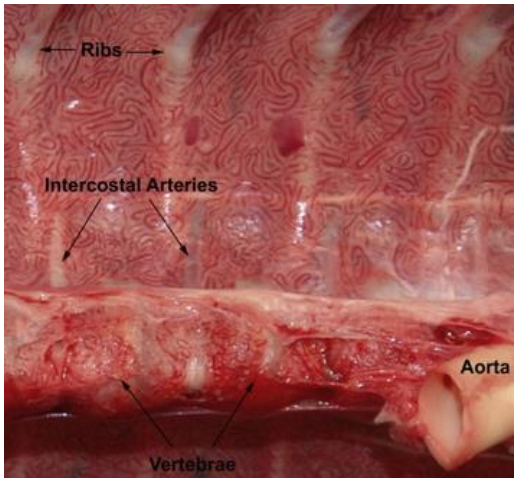

Fig. 1.2 Thoracic *rete mirabile* in a common dolphin (*Delphinus delphi*). From Encyclopaedia of Marine Mammals, 2nd edition, Perrin W., Würsig B., Thewissen J.G.M, Academic Press, 2008.

CHAPTER 2

The nervous system of cetaceans

2.1 Evolution

Along with the above-mentioned morpho-physiological modifications, also the nervous system of cetaceans faced several, though less evident, transformations. Through the analysis of skeletal fossils, it has been possible to partially document the evolution of cetaceans brain starting from their terrestrial ancestors, the archaeocetes. It has to be said that natural endocasts only allow the study of the outer shape and dimension of the brain, leaving some unresolved issues in the understanding of brain evolution in these animals. More recently, computed tomography (CT) has been used to reconstruct the endocranial morphology of fossil cetaceans skulls (Marino et al., 200, 2003). Archaeocetes are a paraphyletic group of primitive terrestrial animals lived from the Early Eocene to the late Oligocene. Several studies demonstrated that some cetaceans' ancestors had smaller brain compared to their evolved modern counterparts, and most of them were characterized by a low encephalization quotient (EQ), defined as the ratio between actual brain mass and predicted brain mass for an animal of a given size (Breathnach, 1955a; Gingerich 1998; Marino et al., 2003; Marino, 2004). Since modern dolphins are characterized by a high EQ (second only to humans), researchers concluded that these animals became highly encephalized during their evolutionary path (Marino et al., 2003). In particular, as occurs for some other mammals, the development results more pronounced in some districts than others. Thus, in toothed whales, it would be more accurate to refer to it as "neocorticalization" (Manger, 2006; Cozzi et al., 2017). On the other hand, baleen whales have a low EQ, even if their large and complex brains suggest an evolutionary transformation, as well (Marino, 2004). As shown in Fig. 2.1, there are many morphological differences between the brains of archaeocetes and modern toothed whales. The most eye-catching ones are the loss of the olfactory peduncles, well developed in archaeocetes, and the great expansion and convolution of hemispheres compared with the ancestors' brain.

Odontocetes undergoes a complete regression of the olfactory bulbs during fetal life (Oelschläger and Buhl, 1985), while in adult mysticetes, olfactory structures, albeit clearly reduced, are manteined (Oelschläger and Oelschläger, 2009).

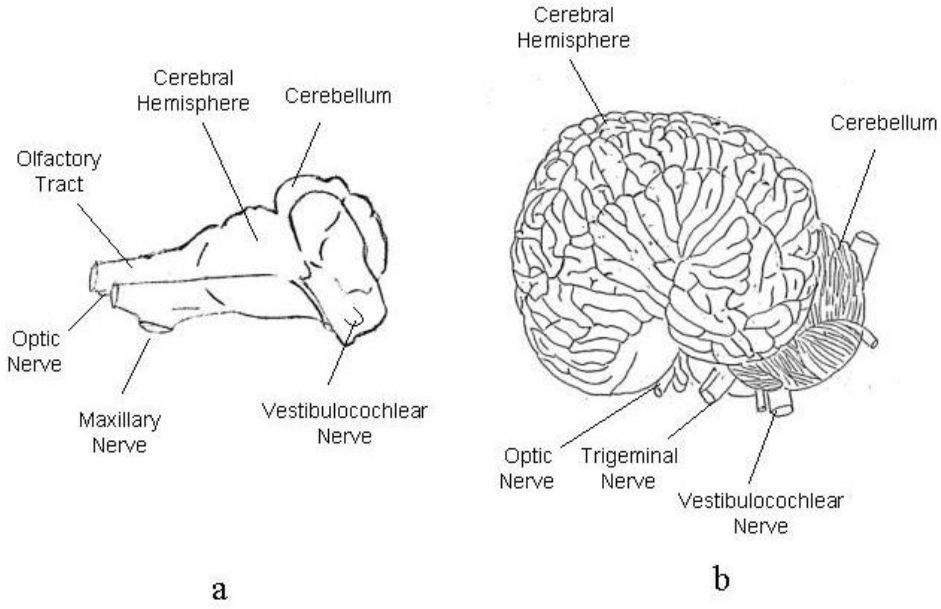

Fig. 2.1 Representation of an archaeocete brain (a) and brain of a contemporary dolphin (b). From Marino, 2004.

Since little change in encephalization was observed during the period that covers the transition from terrestrial to aquatic life, it has been hypothesized that the modifications characteristics of modern cetaceans were the result of a selective pressure occurred after this passage and maybe attributable to other factors (Marino et al., 2000). Different explanations concerning the evolutionary enlargement of cetaceans brains have been proposed. Currently the leading hypothesis is that this increase was the response to ecological and social forces in order to support increasing cognitive abilities (Connor, 2007; Marino et al., 2008). Nevertheless, other questionable theories have been put forward, like the "thermogenesis hypothesis" by Manger

(2006). Manger claims that the large volume occupied by cetacean brain is not related to high cognitive functions and thus to intelligence, but large brains could be efficient thermogenetic organs that counteract heat loss to the water. Manger postulates that these changes probably occurred during oceanic cooling in the Oligocene epoch. Although many hypothesis remain claims in search of scientific evidence, in the last years significant progress has been made in the study of brain size and morphological evolution in cetaceans.

2.2 The central nervous system

As in other mammals, also in cetaceans the central nervous system (CNS) encompasses the brain and the spinal cord. The brain consists of five main subdivisions: medulla oblongata, metencephalon (which comprises pons and cerebellum), mesencephalon, diencephalon and telencephalon. Here the main features and peculiarities of cetaceans CNS are reported, as well as recent findings in gross and microscopic anatomy. The large body of literature mainly concerns delphinids, and in particular the bottlenose dolphin. If data refer to a different species, it will be indicated in the text.

Spinal cord

The spinal cord connects and transmits sensory and motor information between the brain and the periphery of the body. Also in cetaceans, it does not extend the entire length of the vertebral column. In the bottlenose dolphin, it is reported to end about the third lumbar vertebra (Ridgway, 1990). The macro- and microscopical aspect of the dolphin spinal cord reflects the adaptations of the locomotory system. In fact, the minimization of the hind limb is reflected in a reduced lumbar intumescence, when compared with the prominent cervical one, which innervates the pectoral girdle and flippers (Jansen and Jansen, 1969; Cozzi et al., 2017). Histologically, the spinal cord is characterized by a remarkable development of ventral horns, innervating the powerful body musculature. On the other hand, dorsal horns and dorsal columns result quite tiny throughout the entire craniocaudal extension of the spinal cord (Morgane and Jacobs, 1972). This feature has been correlated with a reduction in peripheral sensory inputs (Flanigan, 1966; Cozzi et al., 2017). Adult dolphins lack a spinal central canal, which actually seems to exist in the early prenatal stages of dolphins and whales (Romanes, 1945; Hosokawa, 1955; Flanigan, 1966; Sinclair, 1972). Recent studies (Bombardi et al., 2010, 2013) investigated some neurochemical aspects of the bottlenose dolphin spinal cord. In particular, nitric oxide (NO), substance P (SP) and cholecystokinin (CCK)

were described, pointing out a distribution similar to that reported for other mammalian species. The presence of immunoreactive cells and fibers in different laminae of the spinal cord suggests a role of NO, SP and CCK in spinal sensory and visceral circuitries.

Brainstem

The brainstem consists of myelenccephalon (medulla oblongata), pons and mesencephalon. Myelenccephalon is reported to be very large in comparison with other mammals (Oelschläger and Oelschläger, 2009). This may be due to the considerable development of some cranial nerve nuclei (and their connections), as the trigeminal and the cochlear ones. Malkemper and colleagues (Malkemper et al., 2012) investigated the histologic characteristics of cochlear nuclei of the common dolphin (*Delphinus delphis*) and La Plata dolphin (*Pontoporia blainvillei*). Differently from the ventral cochlear nucleus (VCN), which is very large in these animals, the dorsal cochlear nucleus (DCN) seems to be reduced in size, when compared with what described so far in other mammals. Since in other animals the DCN is responsible for the reflexive orientation of the head and pinnae towards a sound source, authors attributed its reduced size in dolphins to their limited head mobility and the lack of outer ears. The superior olive, considered part of the auditory system and responsible for the directional hearing, is obviously very large in dolphins (Cozzi et al., 2017). The inferior olivary complex recognizes three main subdivisions: the principal nucleus, the dorsal nucleus, and the medial accessory nucleus. In cetaceans this latter component is extremely developed, while the other two subnuclei appear small (Oelschläger and Oelschläger, 2009). The large size of this subdivision may be ascribed to the development of its cerebellar connections (e.g. paraflocculus) and to its putative involvement in a fiber system implicated in directional hearing (Oelschläger and Oelschläger, 2009). It is well known that the vestibular apparatus of dolphins, particularly the semicircular canals, is reduced compared to other mammals and in relation to their body size (Spoor et al., 2002; Spoor and Thewissen, 2008; Kande1 and Hullar, 2010). This finding probably correlates with the limited mobility of the head in these animals. Recently, Kern and

colleagues (Kern et al., 2009) analysed the central vestibular complex in the La Plata dolphin *(Pontoporia blainvillei)* and the common dolphin (*Delphinus delphis*). The vestibular nerve and most of the vestibular nuclei, which receive direct input from the semicircular canals, are reduced in size, whereas the lateral (Deiters') vestibular nucleus was well developed in both dolphin species. The lateral vestibular nucleus of La Plata dolphin showed a size comparable to that of humans, whereas in the common dolphin the nucleus resulted even larger. Since Deiters' nucleus is functionally correlated with cerebellar nuclei, its large size may be related to three-dimensional locomotion. In the mesencephalon of dolphins, there is a very prominent nucleus, named elliptic nucleus, which seems to be an unique structure among mammals, perhaps with the exception of the elephant (Cozzi et al., 2001). The elliptic nucleus is supposed to correspond to an hypertrophied nucleus of Darkschewitsch. Actually, it is unclear whether Darkschewitsch nucleus is integrated into the elliptic nucleus or the two structures perfectly overlap (Oelschläger and Oelschläger, 2009). The large elliptic nucleus, together with well-developed inferior olivary complex and cerebellar structures, seem to play a role in the combination of auditory information with locomotion, thus denoting the importance and supremacy of hearing among other sensory functions in these animals (Oelschlager, 2008). As the elliptic nucleus, also the red nucleus receives a major projection from the cerebellar nuclei and it is well-developed in dolphin. These features (very large elliptic nucleus, red nucleus and also reticular formation), together with the great development of cerebellum, may represent an advantage for the peculiar and exceptional locomotion in the aquatic medium. Odontocetes sleep is unusual and characterized by unihemispheric slow wave sleep (USWS) and suppressed REM phase. This allows the brain to maintain an alternate status of slow-wave sleep and wakefulness in the two hemispheres (Lyamin et al., 2008). The sleep-wake cycle is regulated by different neuronal populations, located in the pedunculopontine and laterodorsal tegmental nucleus (PPT/LDT), locus coeruleus, dorsal and median raphe nucleus, and tuberomammillary nucleus (TMN) (Schwartz and Roth, 2008). Studies investigating this neural system in cetaceans didn't show any striking neuroanatomical difference, compared with terrestrial mammals (Manger et al.,

2003; Dell et al., 2012, 2016a, b). This suggests that, beside physiological differences, the sleep-related neural system in mammals is strongly conserved across species. Nevertheless, both the harbor porpoise (*Phocoena phocoena*) and the minke whale (*Balaenoptera acutorostrata*) present some characteristics that may facilitate the suppression of REM sleep and control the USWS, like an expansion of the peripheral division of the dorsal raphe nuclear complex, an higher number of pontine cholinergic and noradrenergic cells, along with an enlarged posterior commissure (Dell et al., 2016a, b).

Cerebellum

Both odontocetes and mysticetes possess a large cerebellum, although in relation to body mass the cerebellum of baleen whales is not as voluminous as in toothed whales. Among odontocetes, some differences seem to exist. In fact, the sperm whale cerebellum is about 7% of the total brain mass, while the value in the killer whale is almost double (Ridgway and Hanson, 2014). Recently, Hanson and colleagues used magnetic resonance imaging to analyse cerebellar anatomy in the bottlenose dolphin and compare it with human (Hanson et al., 2013). Authors identified and measured the different cerebellar lobules, pointing out the great size of some auditory-associated lobules, like the VIIb, VIII and IX. It is possible that toothed whales, as microchiropterans, have a large cerebellum due to their echolocating abilities and consequently enlarged auditory-associated cerebellar areas (Baron et al., 1996; Maseko et al., 2012; Hanson et al., 2013). Nevertheless, as they claimed, it is premature to assert a functional role without studying connectivity patterns. The flocculonodular lobule, which is the principal terminus of vestibulocerebellar fibers, is logically small in cetaceans, reflecting the rudimentary condition of the vestibular system (Oelschläger and Oelschläger, 2009; Hanson et al., 2013). The paraflocculus is exceptionally volumionous in dolphins. This structure usually receives fibres from the medial accessory inferior olive, which is also particularly large in dolphins, as mentioned before. As reported by some authors (Oelschläger and Oelschläger, 2009; Cozzi et al., 2017), this could be indicative of a functional relationship

between the paraflocculus and the trunk and tail unit. The concomitant great development of the auditory system and cerebellum in toothed whales led Ridgway (2000) to suggest that the cerebellum of odontocetes, among other functions, might be involved in acoustic processing with respect to the localization of objects in water and to three-dimensional navigation.

Diencephalon

As in terrestrial mammals, the diencephalon of cetaceans consists of the epithalamus, the thalamus, the subthalamus, and the hypothalamus. Although the organization of these four structures mostly corresponds with what described in other mammals, some differences exist. The epithalamus includes the pineal gland, whose presence in cetaceans has been largely investigated (Gersch, 1938; Breathnach, 1955b; McFarland et al., 1969; Arvy, 1970; Morgane and Jacobs, 1972; Behrman, 1990; Ridgway, 1990; Duffield et al., 1992; Oelschläger and Kemp, 1998; Oelschläger et al., 2008, 2010; Panin et al., 2012). Despite this large body of literature, the only certain conclusion seems to be the non-constant presence of the pineal gland among cetaceans species and among individuals of the same species, as well. A recent study, performed on bottlenose dolphins, demonstrated the presence of circulating melatonin despite the apparent lack of a pineal gland, suggesting an entirely extrapineal production of the hormone in these animals (Panin et al., 2012). Regarding the thalamus, a noteworthy feature is represented by the extremely large medial geniculate nucleus (MGN), responsible for auditory information relay (Morgane and Jacobs, 1972; Oelschläger and Oelschläger, 2009; Cozzi et al., 2017). The ventral posterior nucleus (VPN) is part of the somatosensory thalamic unit and is thus involved in the transmission of surface and deep sensitivity from the trunk, extremities, and head (Mai and Forutan, 2012). In the bottlenose dolphin, the lateral and the medial subnuclei of the VPN, where the body and the head are represented, respectively, are quite different in size. The medial one is large, compared with its lateral counterpart, suggesting an important somatosensory representation of the head, in contrast to what happens with the body (Oelschläger and Oelschläger, 2009; Cozzi et al., 2017).

Limbic system

Among all the heterogeneous structures constituting the limbic system of cetaceans, the hippocampus deserves special attention. In different cetaceans species a small size of the hippocampus has been reported over the years (Filimonoff 1965; Pilleri and Gihr 1970; Jacobs et al. 1979; Morgane et al. 1980; Schwerdtfeger et al. 1984; Manger 2006; Patzke et al., 2015). In their recent publication, Patzke and colleagues (2015) evaluated hippocampal volume of several mammals, including four cetacean species (harbor porpoise, bottlenose dolphin, Atlantic white-sided dolphin and minke whale). In contrast to all other mammals, including close relatives like the hippopotamus, cetaceans showed a relatively and absolutely small hippocampus, whose volume barely reach the 20% prediction intervals based on the brain size. Furthermore, they observed a completely lack of adult hippocampal neurogenesis in the harbor porpoise and the minke whale, differently from all other mammals examined. Patzke and colleagues hypothesized that the absence of neurogenesis depends on the unusual cetacean sleep, whose "lack of a REM phases throughout the entire life may have led to a cessation of hippocampal neurogenesis". Also the histological aspect of the hippocampus seems to be different in cetaceans. In fact, it is characterized by the lack of the typical convolutions of the *cornus ammonis* and a very small and minimally folded dentate gyrus (Jacobs et al., 1979; Hof et al., 2005). The fact that cetaceans have a small and loosely organized hippocampus, which lack neurogenesis, led some authors to question their presumed cognitive complexity (Patzke et al., 2015). Seen from another perspective, the reduced hippocampus could be correlated to the limited variety of incoming sensory stimuli in animals whose life is auditory-driven. Other structures of the limbic system, like the periarchicortex and the amygdala, are remarkably developed in toothed whales (Schwerdtfeger et al., 1984; Oelschläger and Oelschläger, 2009; Cozzi et al., 2017).

Telencephalon

In cetaceans, and especially in toothed whales, the hemispheres are large and the cortex is especially thin but highly convoluted, compared with other mammals (Morgane at al., 1980). In the bottlenose dolphin, the high development of the cerebral cortex resulted in a lateral rotation/expansion of the entire temporal lobe, as early reported by Jacobs et al. (1979). Most of the telencephalon consists of the voluminous neocortex, while the two other cortices, the archicortex and the paleocortex, are more reduced in dolphins, the latter because of the loss of the olfactory system (Oelschläger et al., 2008; Oelschläger and Oelschläger, 2009). Electrophysiological studies enabled to map motor and sensory projection fields in the cortex of dolphins, pointing out a somewhat different configuration, compared with other mammals (Lende and Akdikmen, 1968; Lende and Welker, 1972; Ladygina et al., 1978; Supin et al., 1978; 2001). The motor area lies rostrally in the frontal lobe and the somatosensory field immediately adjacent to it but a bit more lateral and caudal. Caudally, auditory and visual areas are adjacent and located between the ectosylvian and lateral sulci. Primary and secondary auditory regions are situated more laterally, while visual fields medially (Fig 2.2). Histologically, cetaceans neocortex is thin and characterized by a general loss in granularization, with the complete lack of the internal granular layer (layer IV) (Morgane et al., 1988; Hof et al., 2005; Hof and Van der Gucht 2007; Furutani 2008; Butti et al. 2011). The general layering pattern is characterized by a prominent, thick layer I that is far more cellular than in most terrestrial species, a layer II that contains "extraverted neurons", large clustering pyramidal neurons between layers III and V, and a multiform layer VI (Hof et al., 2005; Butti et al., 2011, 2015). The lack of layer IV, which is the main target of thalamocortical afferents, may suggest a different wiring for thalamocortical projections in cetaceans brain (Revishchin and Garey, 1990; Hof and Van der Gucht 2007). Poth et al. (2005) investigated the neuron numbers in the three sensory cortices of five delphinid species and the pigmy sperm whale (*Kogia breviceps*). Authors pointed out that, in delphinids, increasing brain mass is inversely related to neuron number

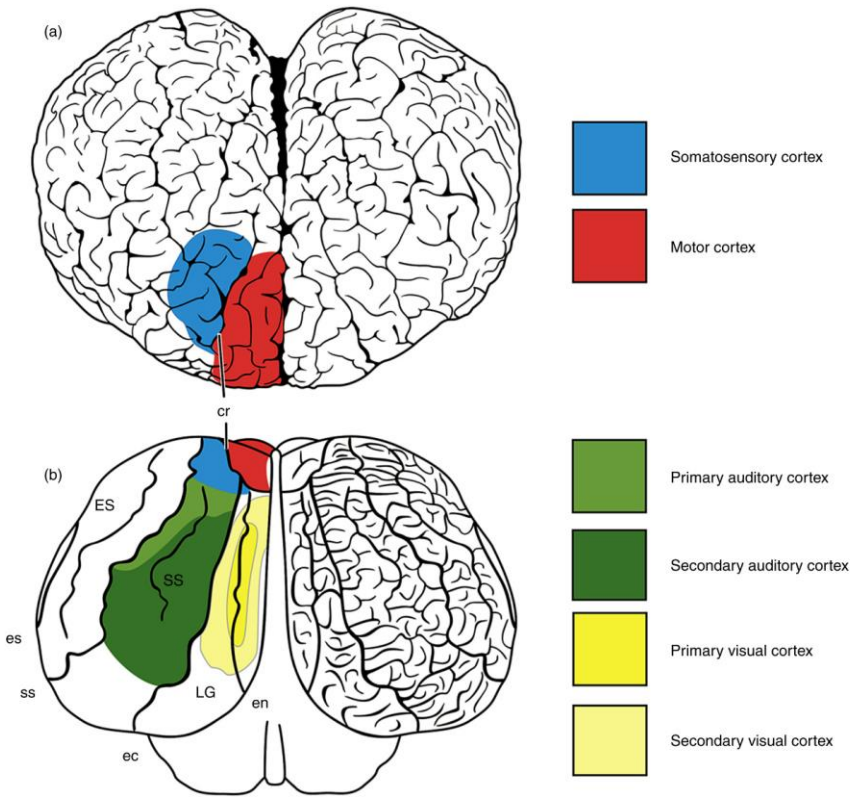

Fig. 2.2. Localization of the primary projection areas in the cerebral cortex of the bottlenose dolphin. Rostral (a) and dorsal (b) view of the dolphin brain. Note the extension of the auditory field in comparison to the other sensory cortices. Abbreviations: cr, cruciate sulcus; ec, lateral sulcus; en, entolateral sulcus; es, ectosylvian sulcus; ES, ectosylvian gyrus; LG, lateral gyrus; ss, suprasylvian sulcus; SS, suprasylvian gyrus. From: Cozzi B., Huggenberger S., Oelschläger HHA., 2017. The Anatomy of dolphins. Insights into body structure and function. Chapter 6. Brain, spinal cord, and cranial nerves. Elsevier, San Diego.

per cortical unit. Furthermore, they did not find any difference between the number of neurons in auditory areas compared with other sensory cortices, suggesting that "an increase in functional significance is associated with the enlargement of the corresponding area, not with a higher neuron number per cortical unit". Von Economo neurons have been observed in layer V of the anterior cingulate, anterior and frontoinsular, and frontopolar cortices of different cetaceans species (Hof and Van der Gucht 2007; Butti et al., 2009). Von Economo neurons, which are suggested to play a role in interoception, sociality, and cognition (Allman et al., 2005, 2010; Craig, 2009) were

originally described in humans, great apes, and later in elephants, and were thought to be unique to only a few species that possess enlarged brains (Nimchinsky et al., 1995, 1999; Hakeem et al., 2009; Allman et al., 2010). Recently, they were identified in the brain of the hippopotamus (Butti et al., 2014), as well as pig, sheep, cow, white-tailed deer, horse and rock hyrax (*Procavia capensis*) (Raghanti et al., 2015). These results suggest that Von Economo neurons were not distinctive of highly encephalized or socially complex species, but their presence among distantly related species may represent convergent evolution of specialized pyramidal neurons (Raghanti et al., 2015).

For several decades, the cytoarchitectonic uniformity and homogeneity of cetacean neocortex raised a problem for views of their cognitive complexity and intelligence. In fact, the complexity of primate brains is thought to lie in the existence of highly modified and differentiated cortical units (Glezer et al., 1988). More recently, in a study carried out on bottlenose dolphins, Hof et al., (2005) identified several features that demostrate the existence of cytoarchitectural diversity in cetaceans. These anatomical observations match the extensive body of evidence for behavioral and social complexity in cetaceans (for a review, see Marino, 2002).

The *corpus callosum*, whose fibres enable the interhemispheric communication, is extremely thin in cetaceans, in relation to their brain mass (Nieto et al., 1976; Ridgway, 1986; Tarpley and Ridgway, 1994). In a more recent study, Keogh and Ridgway (2008) analysed the size and number of fibres along the *corpus callosum* of three odontocetes species. They observed a great heterogeneity of the structure with variations in fibre size and density along its length. Fibres with larger diameter were found near the auditory and visual regions, suggesting a relatively high degree of connection between the parietal lobes and thus underscoring the importance of these sensory systems in the brain of toothed whales. The reduction of the *corpus callosum* in cetaceans seems to indicate a certain independence of each hemisphere from the other, a key feature involved in their typical unihemispheric slow wave sleep. Electroencephalographic studies carried out on bottlenose dolphins reported signs of wakefulness in one hemisphere while the other one was asleep (Serafetinides et al., 1970; Mukhametov et al., 1977; Oleksenko et al., 1992).

2.3 The peripheral nervous system

Unlike the CNS, information regarding the peripheral nervous system (PNS) in cetaceans is scanter and more fragmentary. The great majority of studies concerning the PNS focus on cranial nerves. In mysticetes, and sometimes in the sperm whale (*Physeter macrocephalus)*, the trigeminal nerve is the thickest cranial nerve, while in all other odontocetes the vestibulocochlear nerve is the most conspicuous (and the second largest in baleen whales) (Jacobs and Jensen, 1964). Toothed whales lack the olfactory nerve, which regress during fetal life together with other structures of the olfactory system, except for the persistent and large olfactory tubercle, whose function is still unknown but almost certainly does not concern olfaction (Oelschläger and Buhl, 1985; Cozzi et al., 2017). Nevertheless, a well-developed terminal nerve is present (Johnston, 1914; Sinclair, 1951a, b, 1966; Oelschläger et al., 1987; Ridgway et al. 1987; Demski et al., 1990). Although the functional significance of the terminal system in mammals is still largely unknown, it has been hypothesized that it could serve an autonomic regulation of blood vessels associated with the specialized nasal organ. In fact, the latter uses high-pressure air to produce echolocation and communication sounds and this may probably require peculiar regulatory adaptations in blood vessels (Oelschläger et al., 1987; Ridgway et al. 1987; Demski et al., 1990, Cozzi et al., 2017). In marine dolphins, which possess a well-developed visual system, the optic nerve results thick and its axons very large in diameter (Dawson et al., 1982; Gao and Zhou, 1992; Mazzatenta et al., 2001), but the number of fibres is lower than in terrestrial mammals (Supin et al., 2001). Another interesting difference is the complete decussation of optic nerve fibres. This feature, together with the lateral location of the eyes makes almost impossible for dolphins to have stereoscopic vision. The diameters of the axons tend to be thin in the trigeminal nerve. Morgane and Jacobs (1972) reported a large number of axons in the trigeminal nerve of the bottlenose dolphin, higher than that in human. It has been postulated that this may have to deal with the sensory innervation of the highly specialized blowhole region and its accessory structures (Cozzi et al., 2017). The facial nerve, which, in the bottlenose dolphin, has 7 times more axons than in the human (Morgane and Jacobs, 1972),

provides motor innervation of the blowhole musculature, essential for sonar generation (Mead, 1975; Ridgway, 1990). The great diameter and high number of fibres, which made up the cochlear branch of the eighth cranial nerve emphasise the importance of the auditory system for the life of cetaceans. In contrast, the vestibular counterpart is extremely small, barely reaching one-tenth the diameter of the auditory branch (Gao and Zhou, 1992; Oelschläger and Oelschläger, 2009).

Recently, studies focusing on the neurochemical code of spinal ganglia have been published (Bombardi et al., 2010, 2011). In particular, SP, CCK and NO were described and a possible role of these molecules in the vasomotor control of spinal *retia* was suggested.

The enteric nervous system (ENS) is a fascinating, complex, independent and usually overlooked system, which regulates gastrointestinal functions. Although the digestive system of cetaceans presents many differences in comparison with terrestrial mammals, still few information is currently available on the ENS (Pfeiffer, 1993; Domeneghini et al., 1997; Naka et al., 2007; Russo et al., 2012; Gatta et al., 2014). Domeneghini and colleagues (1997) provided a first general overview on the localization of several neuropeptides (i.e. neuropeptide Y -NPY-, SP, calcitonin gene-related peptide -CGRP-, metenkephalin, gastrin releasing peptide –GRP-, somatostatin and intestinal vasoactive peptide –VIP-) in the gastrointestinal tract of the striped dolphin (*Stenella coeruleoalba*). Although the data reported by Domeneghini et al., mostly resembled what described in terrestrial mammals, some differences were pointed out. For example, the proximal tract of the gastrointestinal system resulted less rich in peptidergic components compared to its distal counterpart, where peptidergic elements were largely represented. Authors hypothesized that this may have to deal with a certain antiperistaltic activity previously reported in cetaceans.

EXPERIMENTAL STUDIES

CHAPTER 3

Distribution of calretinin immunoreactivity in the lateral nucleus of the bottlenose dolphin (*tursiops truncatus*) amygdala

Introduction

The amygdaloid complex is an intriguing and complicated structure that mediates emotional responses to cortical and thalamic inputs and plays a role in generation and consolidation of emotional memories (Aggleton, 2000; LeDoux, 2003; Whalen and Phelps, 2009). The amygdala, located in the medial part of the temporal lobe, is made up of thirteen nuclei, anatomically divided in three groups: deep, superficial and other nuclei. These nuclei establish several connections among themselves and with other brain structures as the hippocampal formation, the cortex, the brain stem and the thalamus (Krettek and Price, 1978; Pitkänen, 2000). The lateral nucleus, which belongs to the deep nuclei group, is the main receiving structure of the amygdala. It collects most of the inputs coming from other brain regions and provides stimuli to other amygdalar nuclei and higher brain structures (Krettek and Price, 1978; Pitkänen, 2000; Sah et al., 2003). In the lateral nucleus, most neurons are represented by type I cells, which are glutamatergic pyramidal neurons; the rest is represented by type II cells, GABAergic non-pyramidal neurons. These latter cells are typically classified in different categories based on the morphology and the expression of neuropeptides and calcium-binding proteins (CBPs). In rat and primate deep amygdalar nuclei, inhibitory interneurons are classically grouped in three morphological classes: polygonal, spherical and fusiform. Polygonal interneurons can be small or big in size and are characterized by several dendritic processes; spherical cells have round somata and few dendrites; and fusiform interneurons present spindle-shaped cell bodies with two dendrites running from the opposite poles of the somata (Kemppainen and Pitkänen, 2000). Type II cells can express CBPs, a family of EF-hand proteins used as a specific marker to identify different neuronal types and their interconnections in the mammalian central nervous system (CNS) (Baimbridge et al., 1992; Hof et al., 1999). CBPs, such as calretinin (CR), modulate the intracytoplasmic concentration of Ca^{2+} and are therefore involved in the regulation of neuronal excitability and synaptic plasticity (Yáñez et al., 2012). Interestingly, in the lateral nucleus of the mouse amygdala, CR shows a distinct spatiotemporal expression pattern of development (Legaz et al., 2005). In the past, the amygdala was considered essential for social

cognition, and lesions at this level were thought to lead to a decrease of social interactions (Dicks et al., 1969; Kling and Cornell, 1971; Kling, 1974, Brothers et al., 1990). More recent studies suggest that lesions to this structure result in an increased social behaviour, in a lack of inhibitions and in decreasing responses to dangerous stimuli (Amaral, 2003; Amaral et al., 2003). These evidences raise the hypothesis that the primary role of the amygdala could possibly be to evaluate potential environmental threats, invalidating the theory of the essential involvement of the nucleus in social cognition. Compared to terrestrial mammals, the CNS of cetaceans presents some morphological and functional differences, which contributed to their adaptation to aquatic environment (Ridgway, 1986, 1990; Marino et al., 2007; Mass and Supin, 2007; Nummela et al., 2007). Therefore, the study of such differences may contribute to speculate on how they affect life in the water. The lateral nucleus, which oversees most afferent inputs from the cortex and thalamus, is expected to be extremely developed in toothed whales, including the bottlenose dolphin (*Tursiops truncatus*). In fact, some structures which project to the lateral nucleus (i.e. the cortex and the acoustic pathways), are highly developed in aquatic mammals. Here we emphasize that sound perception constitutes the main modality through which dolphins perceive the surrounding environment. To the best of authors' knowledge, data on the anatomy of the amygdaloid complex of the bottlenose dolphin are limited (for a general discussion see Cozzi et al. 2017). Interestingly, observations performed in river dolphins suggest that the amygdalar complex in dolphins is larger than in primates (Schwerdtfeger et al., 1984). In the present study, we provide information on the topography, cytoarchitecture and immunohistochemical features (distribution of CR-ir) of the lateral nucleus of the bottlenose dolphin.

Materials and Methods

The entire amygdala of three bottlenose dolphins (*Tursiops truncatus*), belonging to the collections of the Mediterranean marine mammal tissue bank of the Department of Comparative Biomedicine and Food Science (University of Padova, Italy) (website: www.marinemammals.eu;

CITES IT 020), were used. After coding the carcass for freshness, the entire brain was removed, cut into 1cm-thick transverse sections and immediately fixed by immersion in cold 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS; pH 7.4) for at least one week. After tissue fixation, the amygdaloid complex was isolated. After rinsing in PBS, the tissue was cryoprotected in a 30% sucrose solution in PBS (pH 7.4) and stored at 4 °C. Then the tissue was cut on the coronal or sagittal planes into sections having a thickness of 60 µm using a freezing sliding microtome. The sections were stored at 4 °C in a tissue collecting solution (30% sucrose in PBS containing 0.1% sodium azide) until processed for histological and immunohistochemical staining.

Nissl Staining

To help identify the different nuclei of the amygdala and their subdivisions, sections adjacent to immunoperoxidase preparations were stained with thionin. Sections stored in the tissue collection solution were mounted onto gelatine-coated slides and dried overnight at room temperature. Then they were defatted 1 hr in a mixture of chloroform/ethanol 100% (1:1), rehydrated through a graded series of ethanol, stained 30 min in a 0.125% thionin (Fisher Scientific) solution, dehydrated and coverslipped with Entellan (Merck, Darmstaldt, Germany).

Immunoperoxidase

Free-floating sections were washed in PBS (three times for 10 min each). To eliminate endogenous peroxidase activity, the sections were treated with 1% H_2O_2 in PBS for 30 min at room temperature. To block nonspecific bindings, sections were rinsed in PBS (six times for 5 min each) and incubated in a solution containing 10% normal serum (Colorado Serum, Denver, CO, #CS 0922) and 0.5% Triton X-100 (Merck, Darmstadt) in PBS for 2 hr at room temperature. Thereafter, the sections were incubated for 48 hr at 4 °C in a solution containing monoclonal mouse anti-CR antibody (dilution 1:1000; Code N° $6B_3$; SWANT), 1% normal goat serum and 0.5% Triton X-100. After washing in PBS (three times for 10 min each), the sections were incubated in a solution

containing goat biotinylated anti-mouse IgG (diluted 1:200; BA-2000; Vector), 1% normal goat serum and 0.3% Triton X-100 for 2 hr at room temperature. The sections were then transferred to avidin–biotin complex (ABC kit Vectastain, PK-6100, Vector Laboratories, Burlingame, CA) for 45 min, and the immunoperoxidase reaction was developed by 3,30-diaminobenzidine (DAB kit, SK-4100, Vector Laboratories, Burlingame, CA). After washing, the sections were mounted onto gelatine-coated slides, dried overnight at room temperature, dehydrated in ethanol, cleared in xylene, and coverslipped with Entellan (Merck, Darmstaldt, Germany).

Antibody specificity

The amino acid sequence of CR of the bottlenose dolphin had already been compared with those of other mammals, showing a correspondence over 93% (Cozzi et al., 2014). The secondary antibody specificity was tested through the substitution of the primary antiserum with PBS, resulting in an abolished staining.

Analysis of Sections

Using a microscope Zeiss Axioplan (Carl Zeiss, Oberkochen, Germany), the main subdivisions of the lateral nucleus in Nissl stained sections were analysed. In immunohistochemical sections CBP-ir neurons were observed and gathered in four groups depending on their morphology (polygonal, spherical, fusiform, pyramidal) and the number and characteristics of the dendrites. To each cell, a grade of immunoreactivity (weak, moderate or strong) was also assigned, and the perikaryal area was measured (only in neurons with an evident nucleus and nucleolus).

Results

General topography and subdivisions

In the bottlenose dolphin, as in primates, the high development of the cerebral cortex resulted in a lateral rotation/expansion of the amygdala and the entire temporal lobe, as reported by Jacobs et al. (1979). The lateral nucleus was delimited laterally by the external capsule and it was positioned ventrally to the central nucleus, dorsally to the paralaminar nucleus and laterally to the basal nucleus, from which it was separated by a bundle of fibres. The lateral nucleus, observable in every coronal section from the rostral to the caudal end, was the most developed structure of the amygdaloid complex of the bottlenose dolphin and it reached an area of 0.9-1 cm^2 at its point of maximal extension. The histological staining allowed us to recognize six main subdivisions within the lateral nucleus: lateral, ventromedial, dorsomedial, central, intermediomedial and paracapsular. The lateral subdivision was the largest area occupying the entire rostrocaudal extension of the lateral nucleus (Fig. 3.1 A-F). In the rostral part of the amygdala, the lateral subdivision formed the entire lateral nucleus. Neurons in the lateral subdivision had a variety of shapes and sizes. The neuronal packing density in this subdivision was lower than in the ventromedial and paracapsular subdivisions (Fig. 3.2 A). The ventromedial division appeared as an elongated structure located in the ventromedial portion of the lateral nucleus (rostral and medial parts), near the basal nucleus, from which it is separated by means of a bundle of fibres (Fig. 3.1 A-E). This subdivision appeared homogeneous and contained medium to large sized neurons (Fig. 3.2 B). The dorsomedial subdivision occupied the lateral part of the lateral nucleus throughout its rostrocaudal extent; however, this subdivision was not observed in the rostral and caudal poles of the nucleus (Fig. 3.1 A-F). This subdivision had cytoarchitectonic features like those of the lateral subdivision (Fig. 3.2 C). The dorsomedial subdivision could be easily identified since the fibre bundles between the dorsomedial division and the lateral division of the lateral nucleus, and those between the basal nucleus (magnocellular division) and the central nucleus, were relatively broad and well evident.

The bundle of fibres that separated the dorsomedial division from the lateral one was particularly thick in the medial part of the amygdala but progressively disappeared toward the caudal end. The central division was located dorsally to the ventromedial division and medially to the lateral division (Fig. 3.1 A-D). The neurons of the central division were quite large and darkly stained (Fig. 3.2 D). However, the border between the central division and the lateral division was difficult to identify. The intermediomedial subdivision, observed only in the caudal portion of the amygdaloid complex, was located dorsally to the ventromedial division and appeared as a protrusion of the lateral nucleus inside the basal nucleus (Fig. 3.1 E). Neurons in the intermediomedial division had a variety of shape and size and were lightly stained (Fig. 3.2 E). The paracapsular subdivision, located in the caudal half of the amygdala, was a sort of a bundle of cells located laterally to the lateral subdivision (Fig. 3.1 D-F). This subdivision consisted of two parts, one included between the fibres of the external capsule and the other one contiguous to the lateral subdivision. The paracapsular subdivision showed a high density of darkly-stained neurons (Fig. 3.2 F). Occasionally, intercalated cell masses can be observed as little clusters of small neurons placed between the fibre bundles, which separate the lateral and the dorsomedial subdivisions.

CR-immunoreactivity

In all three individuals, a strong CR-ir has been observed in fibres and neurons of the lateral nucleus (Fig. 3.3 A, B). The great majority (95.3%; 364/382 cells; n=3) of stained cells belonged to the non-pyramidal class of neurons. Three types of immunoreactive non-pyramidal neurons were observed: polygonal, spherical and fusiform. Polygonal neurons had angular cell bodies of variable sizes, with at least three primary dendrites of variable thicknesses emanating from the soma (Fig. 3.3 C-G). Spherical neurons had a roundish somata and very thin primary dendrites (Fig. 3.3 H). Fusiform neurons had spindle-shaped somata with dendrites typically emanating from opposite poles of the cell body (Fig. 3.3 I, J). Among non-pyramidal immunoreactive cells, polygonal neurons were the most representative group (80.5%; 293/364 cells; n=3), followed by spherical

(13.46%; 49/364 cells; n=3) and fusiform (6.04%; 22/364 cells; n=3) neurons. The perikaryal area, expressed as median and interquartile range (IQR), was measured for every stained neuron with a visible nucleus (Table 3.1). Based on the observation of neurons trough the microscope and on a rough evaluation of the measures obtained, polygonal cells seemed to belong to two dimensional subclasses (small and large sized) but further statistical analysis would be needed to confirm. Immunoreactive neurons showed different grades of staining intensity. Spherical cells, and–some polygonal cells were the most intensely stained elements, while pyramidal neurons showed a faintly CR-immunoreactivity. Only the 4,7% (18/382 cells; n=3) of CR-ir cells could be classified as pyramidal cells. These neurons were characterized by a large, lightly stained pyramidal cell body (Fig. 3.3 K). The neuropil appeared strongly marked in the lateral nucleus, with darkly stained fibres running through it (Fig. 3.3 L). CR-ir did not show any specific distribution pattern and stained elements were present in all parts and subdivisions of the lateral nucleus at any level. Clusters of small roundish or polygonal darkly stained and densely packed cells were observed covering the ventral surface of the lateral nucleus. These immunoreactive "granular islands" probably represented intercalated masses.

Discussion

In recognition of their close phylogenetic relationship, mammals formerly classified as Cetaceans and Artiodactyls, have been recently grouped together into the clade Cetartiodactyla (Montgelard et al., 1997; Gatesy et al., 1999; Matthee et al., 2001; Gingerich et al., 2001; Thewissen et al., 2007; Rubes et al. 2012). Unfortunately, at the best of our knowledge, only few data have been published on the cytoarchitectonic organization of the lateral nucleus of terrestrial Cetartiodactyla (Lauer, 1982). Therefore, here we compare the results obtained in the bottlenose dolphin with rat, human and monkey, which represent the species in which the relative size of the amygdala is more comparable to that of dolphins. The structure of the amygdala in the bottlenose dolphin closely resembles that of other mammals and comprises deep, superficial and others

nuclei/areas. The large size of the amygdaloid complex may be related to the multisensory function of this structure. In fact, in terrestrial mammals, the amygdala receives olfactory, gustatory, somatosensory, visual, and auditory input. In this respect, the auditory system may have a particularly high representation in the amygdala of bottlenose dolphins. The remarkable size of the deep nuclei, including the lateral nucleus, could be related to the considerable development of the temporal lobe and to audition (Morgane and Jacobs, 1972; Stephan and Andy, 1977). Interestingly, the superficial nuclei in the anosmatic harbor porpoise have the same proportion in the whole amygdaloid complex as in the macrosmatic sheep (Cozzi et al., 2017). Our observations in the bottlenose dolphin confirm the general topography of the lateral nucleus described in primates. Laterally, it is delimited by the external capsule and it is positioned ventrally to the central nucleus, dorsally to the paralaminar nucleus and laterally to the basal nucleus. However, a relevant difference is represented by the great number of subdivisions. In fact, while the literature reported only two subdivisions in human (Sorvari et al., 1995; Pitkänen and Kemppainen, 2002), four in monkey (Pitkänen and Amaral, 1998; Pitkänen and Kemppainen, 2002) and three in rat (Pitkänen et al., 1995; Pitkänen and Kemppainen, 2002), we identified six cytoarchitectonic subdivisions with one of them further separable in two parts (inner and outer). As other authors suggest, in the human lateral nucleus more subdivisions will be probably identified with additional detailed studies (Sorvari et al., 1995; Pitkänen and Kemppainen, 2002). The lateral nucleus receives numerous sensory (somatosensory, acoustic, visual, visceral) and mnemonic stimuli that are integrated trough interdivisional and intradivisional connections (Pitkänen et al., 1995, 1997; Pitkänen and Amaral, 1998; Pitkänen and Kemppainen, 2002). In the bottlenose dolphin, the presence of a high number of subdivisions could reveal an outstanding capability of elaboration. It could be hypothesized that the remarkable size of this nucleus, together with the high number of subdivisions identified, could increase the ability of the bottlenose dolphins to elaborate and confer emotional significance to external stimuli. In the lateral nucleus of the bottlenose dolphin, almost all CR-ir neurons (80%) are represented by polygonal cells of small and large size. This result contrasts in part with what

described in human (Sorvari et al., 1996), rat (McDonald, 1994; Kemppainen and Pitkänen, 2000) and monkey (McDonald, 1994) where the majority of CR-ir cells of the lateral nucleus consists of small spherical or bipolar/bitufted neurons. However, in comparison with the lateral nucleus of rat, a higher number of large multipolar stained neurons have been described in monkey (McDonald, 1994). In addition, while in dolphins we did not recognize any area or subdivision more intensely labelled or with more abundant immunoreactive cells, in rat and primates such difference has been described. In fact, as reported by other studies, the subdivisions showing more CR-ir elements are the medial one in humans, the ventral one in monkey, and the ventrolateral one in rat (McDonald, 1994; Sorvari et al., 1996; Kemppainen and Pitkänen, 2000). As described in other species, a low percentage (almost 5%) of lightly stained, pyramidal cells, which resemble projecting neurons, shows CR-immunolabelling. In the bottlenose dolphin, polygonal and spherical immunoreactive neurons show a mean size which is intermediate between the values obtained in rat and in human (Pitkänen and Kemppainen, 2002), while fusiform CR-ir cells result larger in dolphin than in the aforementioned species. The deep nuclei of the amygdala play a critical role in the regulation of the emotional behaviours including fear and anxiety (Adamec et al., 2011; Zangrossi et al., 1999). Interestingly, alterations in CBPs expression contribute to the genesis of anxiety behaviours in rats (Yilmazer-Hanke et al., 2002). In the lateral nucleus of the human amygdala, CR-ir neurons form symmetric inhibitory synapses on the somata and proximal dendrites of calbindin-D28k-ir inhibitory interneurons. Since calbindin-D28k-ir interneurons make inhibitory synapses on the pyramidal cells, the final goal of the CR-ir interneurons could be the synchronization of cells activity (Sorvari et al., 1998). CR-ir neurons in the lateral nucleus of the dolphin amygdala are mainly non-pyramidal inhibitory neurons, which may play an important role in the control of information flowing in the nucleus.

In conclusion, here we provide the first description of the lateral nucleus of the amygdala of the bottlenose dolphin, presenting data on its cytoarchitectonic and chemoarchitectonic properties in comparison with other species. Since the comparative anatomical analysis represents the first step

for comprehension, combining these data with studies on neural connections could hopefully lead to discover functional differences and similarities with respect to other species.

TABLE 3.1. Perikaryal areas of CR-ir neurons in the lateral nucleus of the bottlenose dolphin amygdala.

morphotype	**area µm^2**	**min µm^2**	**max µm^2**
polygonal	186.2 (IQR=140.5-250.7)	56.8	511.2
spherical	83.67 (IQR=73.45-104.2)	46.40	170.7
fusiform	126.8 (IQR=98.73-168.8)	77.46	238.5
pyramidal	284.8 (IQR=247.8-318.6)	179.7	477

Figures

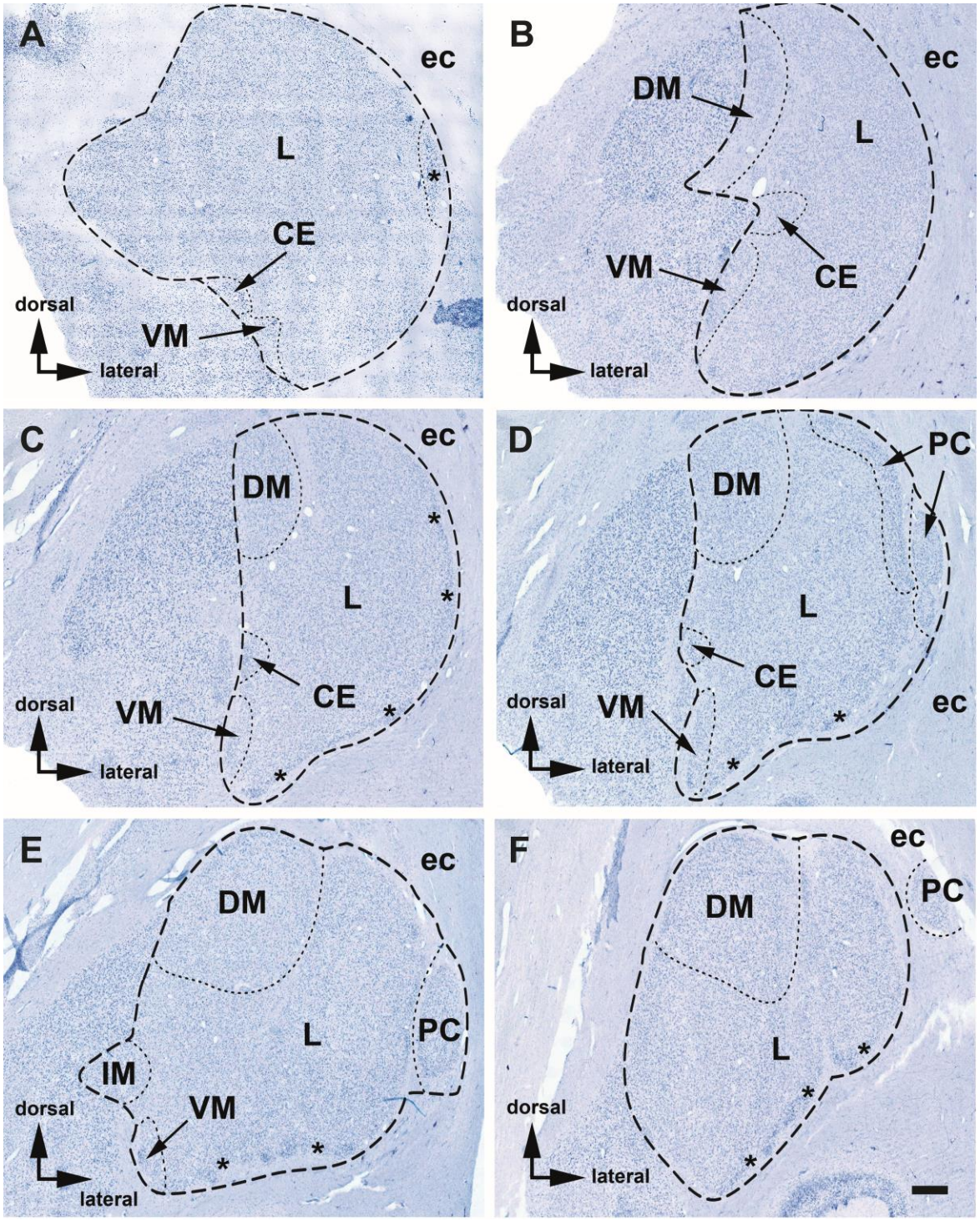

Fig. 3.1. Brightfield photomicrographs from thionin-stained coronal sections showing the topography of the lateral (L), ventromedial (VM), dorsomedial (DM), central (CE), intermediomedial (IM) and paracapsular (PC) subdivisions. The six sections are arranged from rostral (A) to caudal (F). Asterisks indicate the paralaminar nucleus. Abbreviation: ec, external capsule. Scale bar = 1000µm in F (applies to A-F).

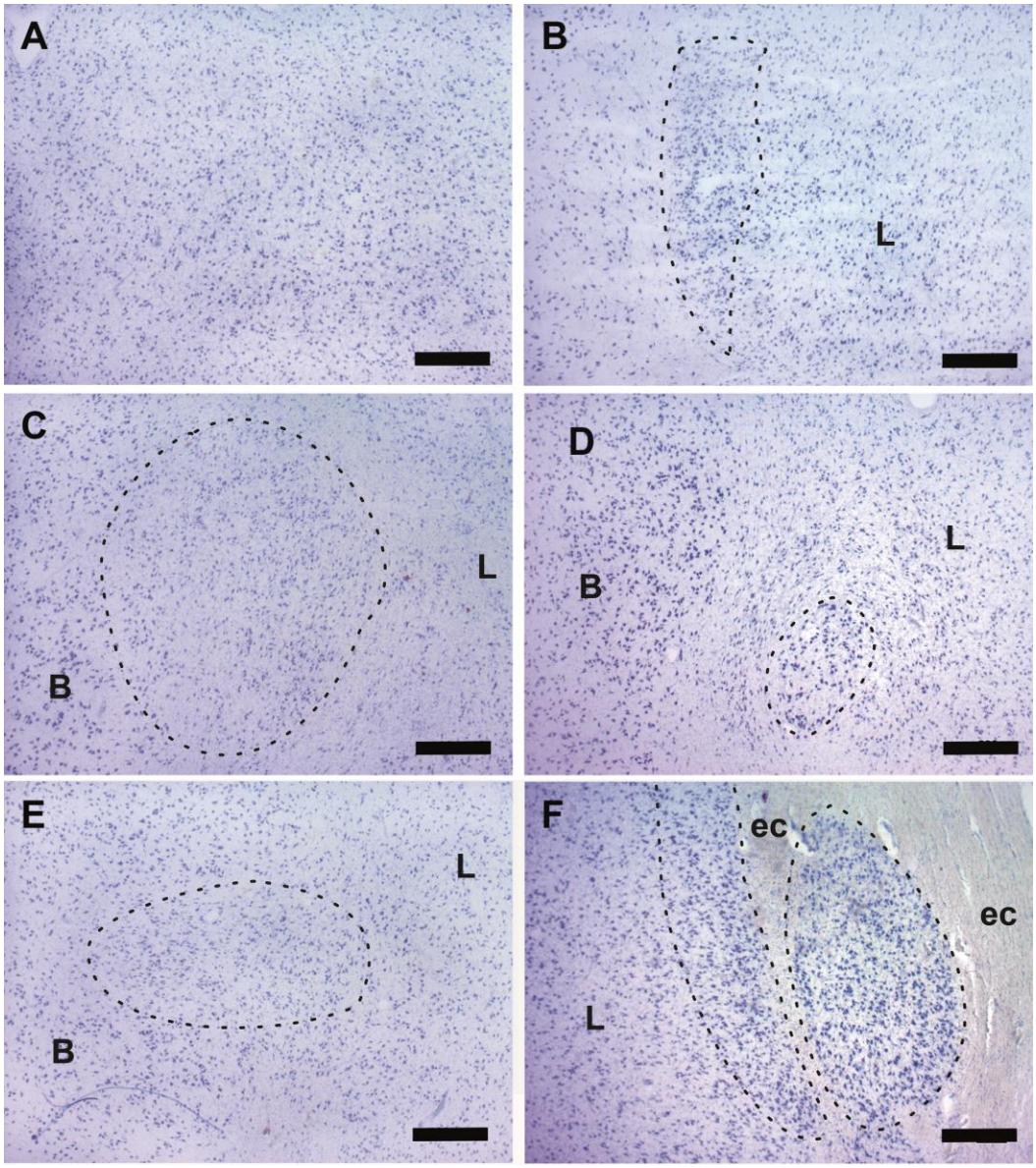

Fig. 3.2. Brightfield photomicrographs of thionin-stained transverse sections showing the unique cytoarchitectonic features from the different subdivision of the lateral nucleus. (A) The lateral subdivision is composed of neurons with different shapes and sizes. (B) The ventromedial subdivision (bordered by dashed line) contains neurons that are medium to large in size. (C) The dorsomedial subdivision (bordered by dashed line) has cytoarchitectonic features very similar to those of the lateral subdivision. (D) The central division (bordered by dashed line) shows large and darkly stained neurons. (E) The intermediomedial division (bordered by dashed line) exhibits lightly stained neurons with different shape and size. (F) The paracapsular division (bordered by dashed line) is characterized by a high density of darkly-stained neurons. Abbreviations: B, basal nucleus; L, lateral subdivision of the lateral nucleus; ec, external capsule. Scale bar = 500 μm (applies to A-F).

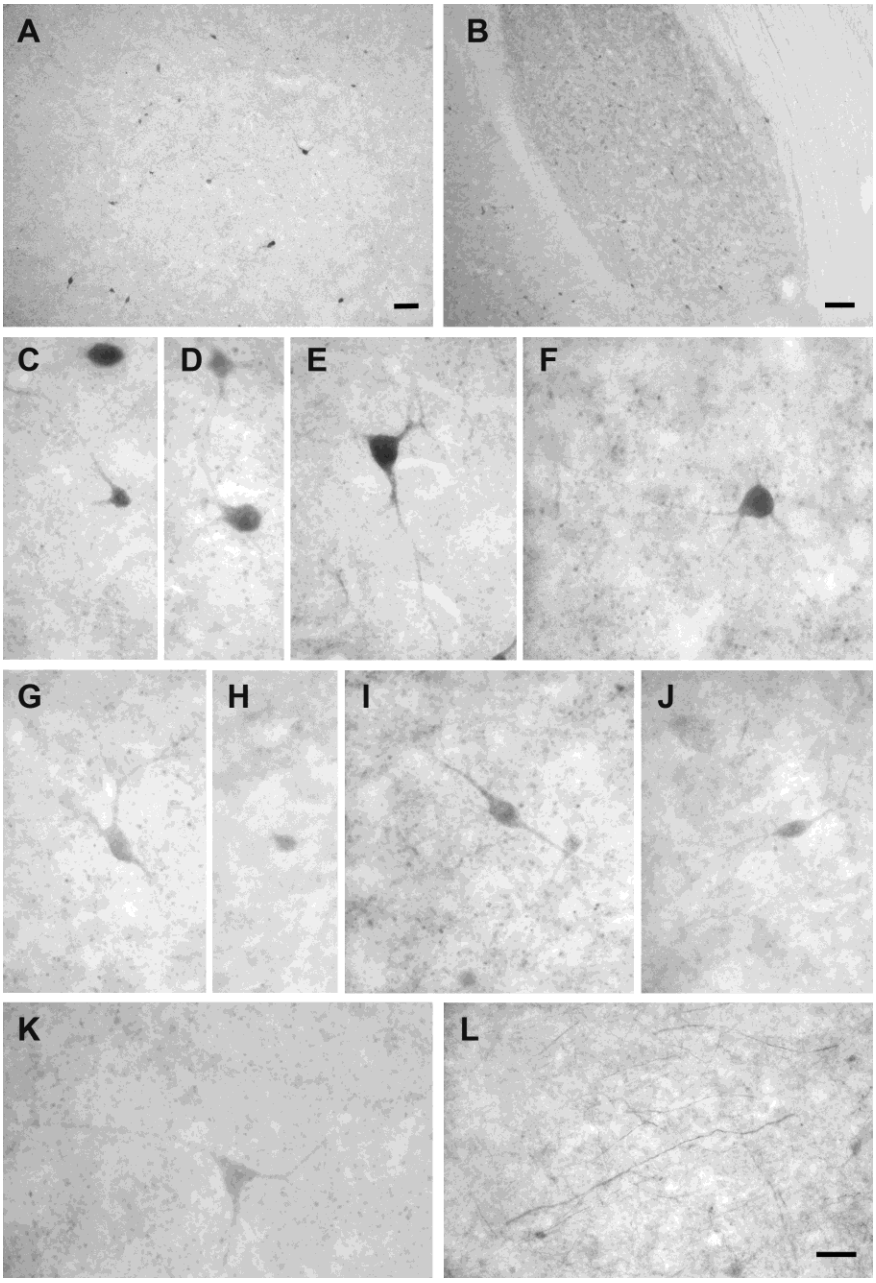

Fig. 3.3. Brightfield photomicrographs of transverse sections showing the various type of calretinin immunoreactive neurons in the lateral nucleus. (A, B) General distribution of immunopositive neurons in the lateral (A) and paracapsular (B) subdivisions. (C-G) Polygonal non-pyramidal neurons have medium to large angular somata that emanate primary dendrites with variable thickness. (H) Spherical non-pyramidal neuron has a small roundish cell body and very thin primary dendrites. (I, J) Fusiform non-pyramidal neurons has a spindle-shaped soma that emanates primary dendrites from its opposite poles. (K) Pyramidal neurons that shows a large lightly stained pyramidal cell body. (L) Neuropil contains darkly stained fibres. Scale bar = 20 μm in F (applies to A-F).

CHAPTER 4

Calcitonin gene-related peptide (cgrp) expression in the spinal cord and spinal ganglia of the bottlenose dolphin (*tursiops truncatus*)

Introduction

The spinal nerves, with their dorsal and ventral roots, carry sensory and motor inputs from the spinal ganglia and from motor neurons of the spinal ventral horn, respectively (Todd, 2010). Architecturally, the gray matter of the spinal cord is classically organized in ten layers extended throughout the cord, named Rexed's laminae. The classification is based on neuronal size, shape, cytological features, and density. Spinal ganglion (SG) neurons are a heterogeneous population of cells that receives a wide variety of sensory information of mechanical, thermal, chemical and noxious origin (Lallemend and Ernfors, 2012). Depending on the type of information carried, SG neurons send their axonal process to different laminae of the spinal cord dorsal horn (Todd, 2010). The spinal cord of the bottlenose dolphin, in comparison with other mammals, has small dorsal horns, (Morgane and Jacobs, 1972; Cozzi et al., 2017). Furthermore, in this animal, the dorsal roots are reported to be thin and lesser developed than the ventral ones. Some authors hypothesize that there might be a correlation between these features and a general loss of sensory information (including nociception), especially from the reduce extremities of their body (Cozzi et al., 2017). Calcitonin gene-related peptide (CGRP), a 37-amino acids peptide encoded trough alternative splicing of the calcitonin gene, is a neuropeptide mainly synthesized by motor neurons of the spinal cord and trigeminal and spinal ganglion neurons, and released by their terminals (Amara et al., 1982; Rosenfeld et al., 1983). CGRP carries out a wide variety of biological functions including vasodilatation, neurogenic inflammation (McCullock et al., 1986; Van Rossum et al., 1997; Kristiansen and Edvinsson, 2010) and transmission of nociceptive information (Van Rossum et al., 1997; Yu et all 2009). Immunohistochemical studies demonstrate a widespread distribution of this peptide in both peripheral and central nervous system of mammals (Gibson et al., 1984; Skofitsch and Jacobowitz 1985; Hökfelt et al., 1992; Batten et al., 1989; McNeill et al., 1988; Conrath et al., 1989; Van Rossum et al., 1997). In the spinal cord of several species, CGRP-immunoreactive (-ir) fibres and neurons are distributed throughout the entire length of the structure. Immunolabeled fibres are extensively distributed at any level of the spinal cord, but in the sacral segments their

number is greater, depending on the increase of the gray:white matter ratio (Gibson et al., 1984). The CGRP-ir somata are localized in laminae I-II of the spinal cord where they act as interneurons and nociceptive specific cells receiving the unmyelinated-C and myelinated-Aδ fibres from the SG (Harmann et al., 1988; Gibson et al., 1984). In the ventral horn, the peptide has been found in motor neurons where it shows a specific granular pattern (Conrath et al., 1989; Skofitsch and Jacobowitz 1985; Gibson et al., 1984; Van Rossum et al., 1997). In SG the 40%-50% of primary afferent neurons, especially small and medium sized cells with a slow conduction velocity, has been shown to be CGRP-ir (Gibson et al., 1984, Ju et al., 1987; Levine et al., 1993; Van Rossum et al., 1997); these cells generally contain also other peptides such as substance P (SP) and cholecystokinin (CCK) (Wiesenfeld-Hallin et al., 1984; Gibbins et al., 1987; Oku et al., 1987). The involvement of CGRP in pain transmission and regulation has been clearly demonstrated: through its neuronal excitatory effect, together with other neuropeptides, CGRP enables the nociceptive information to reach the dorsal horn of the spinal cord from where it is transmitted to the brain by projection neurons (Neugebauer et al., 1996; D'Mello and Dickenson 2008; Yu et al., 2009). In SG, CGRP acts also as a neuromodulator: it potentiates the role of other compounds such as SP, retarding its enzymatic degradation (Oku et al, 1987; Levine et al., 1993; Van Rossum et al., 1997). Sensory neurons, especially nociceptive sub-populations, are generally grouped in two classes, peptidergic and non-peptidergic. CGRP represents, together with SP, one of the most used markers of the peptidergic class. On the other hand, non-peptidergic neurons are usually identified as isolectin B4 (IB4) binding cells, at least in the mouse. The majority of studies on the localization and function of neurotransmitters in the spinal cord and spinal ganglia has been carried out on laboratory animals. Data on large mammals are scant (Bombardi et al., 2010, 2011, 2013; Russo et al., 2010, 2011, 2013; Dudek et al., 2016) and to the best of authors' knowledge no data are available on the distribution of CGRP in the spinal cord and SG of Cetaceans. Thus, the focus of the present research is to determine the distribution of CGRP immunoreactivity in the SG and the spinal cord of the bottlenose dolphin (*Tursiops truncatus*). A preliminary analysis on CGRP- and SP-

immunoreactivity and affinity to IB4 was performed on cervical, thoracic, lumbar and caudal segments of spinal cord and corresponding SG. Subsequently, the caudal tract was examined in depth, describing and quantifying CGRP-IR neurons and their co-localizations with SP. In this way, we could add to our previous researches (Bombardi et al., 2010, 2011, 2013) new information concerning the neurochemical code of the spinal cord and spinal ganglia in Cetaceans.

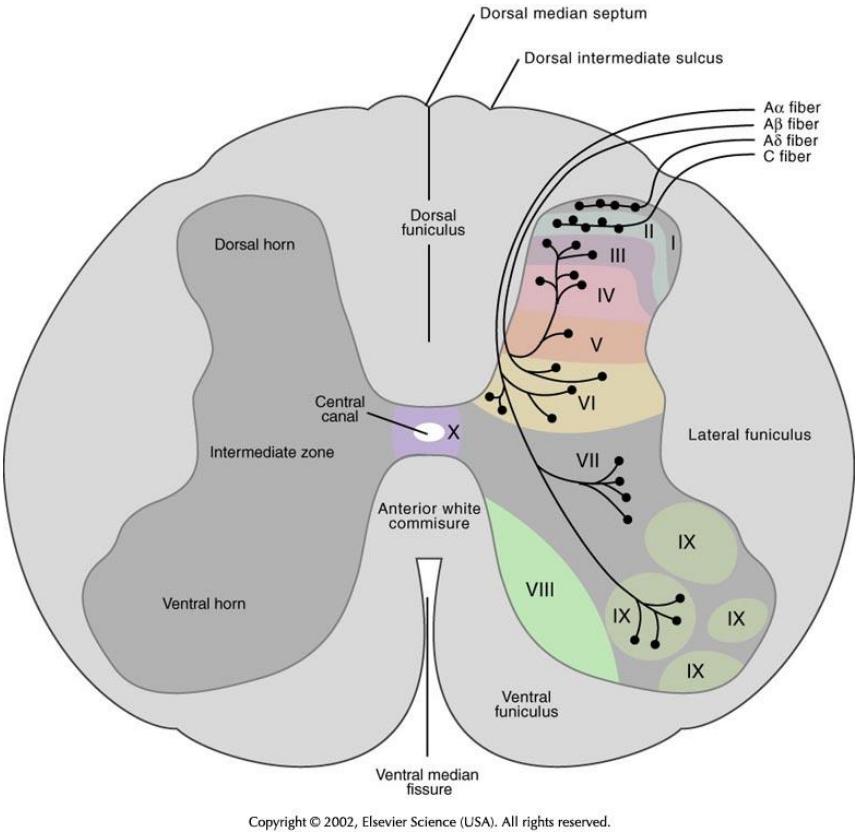

Fig. 4.1 Spinal Cord input from dorsal root ganglio, and representation of Rexed's laminae

Materials and methods

Spinal cords and SG of bottlenose dolphins (*Tursiops truncatus*) were kindly provided by the Mediterranean marine mammal tissue bank of the Department of Comparative Biomedicine and Food Science of the University of Padova (Italy) (MMMTB, www.marinemammals.eu). The MMMTB (CITES IT020) works under the auspices of the Italian Ministry for the Environment and

the University of Padova, and receives tissue specimens from cetaceans stranded along the Italian coasts of the Mediterranean, or directly samples tissues from dolphins and whales brought to its facilities for post-mortem diagnosis. All carcasses are coded for freshness. Once removed, tissue fragments were washed in PBS (0.1 M phosphate buffer saline, pH 7.4) and immersed in 4% buffered formalin for at least 24 h at 4 ° C. After rinsing in PBS, tissues were cut, transferred to a mixture of PBS–sucrose–azide and OCT compound (Tissue Tek®, Sakura Finetek Europe, Alphen aan den Rijn, the Netherlands) at a ratio of 1:1 (overnight), and then embedded in 100 % OCT. Tissue was subsequently mounted in Tissue Tek® mounting medium, frozen and sectioned at 15 µm on a cryostat. Cryosections were collected on poly-L-lysine–coated slides and processed for histological and immunohistochemical labelling.

Nissl Staining

To better identify the laminae of the spinal cord, and in order to obtained area measurements of motor neurons and neurons of the spinal ganglia, cryosections adjacent to immunoperoxidase preparations were stained with 0.125% cresyl-violet solution for 10 minutes, dehydrated and coverslipped with Entellan (Merck, Darmstaldt, Germany).

Immunoperoxidase

After washing in PBS, cryosections were treated with 1% H_2O_2 in PBS for 30 minutes at room temperature to eliminate endogenous peroxidase activity. To block nonspecific binding, sections were rinsed in PBS (six times for 5 minutes each) and incubated in a solution containing 10% normal goat serum (Colorado Serum, Denver, CO, #CS 0922) and 0.5% Triton X-100 (Merck, Darmstadt) in PBS for 2 hours at room temperature. Thereafter, the sections were incubated in a solution containing rabbit polyclonal antibody anti-CGRP (1:8000; C8189; Sigma-Aldrich) for 48 hours at 4°C. The primary antibody was diluted in 0.01 M PBS with the addition of 0.1% sodium azide, containing 1% normal goat serum and 0.5% Triton X-100. After rinsing in PBS, the sections

were incubated in secondary antibody solution (see Tab. 4.2 for antibody details) for 2 hours at room temperature in a secondary antibody solution containing 1% normal goat serum and 0.3% Triton X-100, diluted in PBS. The sections were then transferred to avidin–biotin complex (ABC kit Vectastain, PK-6100, Vector Laboratories, Burlingame, CA) for 1 hour, and the immunoperoxidase reaction was developed by 3,30-diaminobenzidine (DAB kit, SK-4100, Vector Laboratories, Burlingame, CA). After rinsing, the sections were counterstained with cresyl-violet, dehydrated in ethanol, cleared in xylene, and coverslipped with Entellan (Merck, Darmstaldt, Germany).

Immunofluorescence

Cryosections were washed in PBS and processed for double immunostaining. To block non-specific bindings, the sections were incubated in a solution containing 20% normal goat serum (Colorado Serum Co., Denver, CO, USA) and 0.5% Triton X-100 (Merck, Darmstadt, Germany) in PBS for 2 hours at room temperature (RT). Where immunohistochemical staining for IB4 (biotin conjugate) was performed, sections were incubated for 15 minutes in avidin solution, rinsed for 5 minutes in PBS, and incubated for 15 minutes in biotin solution (Avidin/Biotin Blocking Kit, cat. No. SP-2001, Vector, Burlingame, CA). Thus, background staining due to endogenous avidin-binding activity was avoided. The cryosections were incubated overnight in a humid chamber at RT with a cocktail of primary antisera (Tab. 4.2) diluted in 1.8% NaCl in 0.01M PBS containing 0.1% sodium azide. After washing in PBS (3x10 minutes), the sections were incubated for 1 hour at RT in a humid chamber in a mixture of two secondary antibodies (Tab. 4.2) diluted in PBS. Section processed for IB4 staining were additionally incubated in a streptavidin Texas Red-conjugated PBS solution. Then the cryosections were rinsed in PBS and incubated with the blue fluorescent Nissl stain solution (NeuroTrace®, Molecular Probes, Eugene, OR, USA) for 40 minutes at RT in order to identify the neurons. Section were finally mounted in buffered glycerol at pH 8.6.

Analysis

As the number of samples is limited, the qualitative results reported below refer to all the spinal tracts examined, whereas quantitative information (areas and percentages of immunoreactive motor neurons of the spinal cord and neurons of the SG) have been collected only from the caudal compartment. Sections stained using cresyl-violet and immunoperoxidase technique were analyzed with a microscope Zeiss Axioplan (Carl Zeiss, Oberkochen, Germany). Mean area of motor neurons and SG neurons were measured in five histological and five adjacent immunoperoxidase-stained sections for each animal. Double immunofluorescence staining preparations were analyzed with a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes. The images were recorded with a DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). The number of SP- and CGRP-ir neurons was expressed as relative percentage (± standard deviation) while area measurements were expressed as median and interquartile range (IQR). The number of size categories in which SG neurons were to be subdivided, and the definition of the limits for each category were defined through statistical analysis. To determine the exact distribution of neurons into classes, a cluster analysis was performed using K-means algorithms. To determine the exact number of cluster, a scree plot was set up.

Results

Nissl staining

The motor neurons of the caudal spinal cord of the bottlenose dolphin have a mean area of 1772 μm^2 (IQR= 1345-2171), while neurons of the caudal SG display a mean area of 2022 μm^2 (IQR= 1295-3101). Statistical analysis of area measurements points out the existence of four subpopulations of SG neurons, namely small (< 1660 μm^2), medium (1660-2800 μm^2), large (2800-4200 μm^2) and giant (> 4200 μm^2).

Immunoperoxidase

In the spinal cord, the same pattern of CGRP immunoreactivity is observed at any level. CGRP-ir fibres and terminals are most concentrated in laminae I and II and Lissauer's tract where they show a strong immunoreactivity with punctate staining. Few darkly stained fibres are also localized in lamina X throughout the entire spinal cord (Fig 4.1 A-B). Between ir-fibres of the laminae I-II, few CGRP-ir small cells are occasionally observed (Fig 4.1 C). In the ventral horn, the majority of motor neurons of lamina IX show a somatic staining with the typical granular pattern already described for CGRP- immunoreactivity (Conrath et al., 1989; Skofitsch and Jacobowitz 1985; Gibson et al., 1984; Van Rossum et al., 1997) (Fig. 4.1 I-J). In the thoracic tract, neurons of the lateral grey column show CGRP-ir, with the same characteristics outlined for ventral horn motor neurons. In the ventral horn CGRP-ir cells represent the 81 ± 4.6 % (543/663 cells; n=3) of total motor neurons population and display a mean area of 2002 μm^2 (IQR=1624-2373). CGRP-ir primary afferent neurons of SG belong mostly to medium and small subclasses and show an intense somatic staining at any level (Fig. 4.1 D-H). In caudal SG, CGRP-ir neurons account for the 40 ± 3.7 % (744/1896 cells; n=3) of the neural population, with a mean area of 1518 μm^2 (IQR=1150-1915).

Immunofluorescence

In the spinal cord, CGRP and SP result co-localized in lamina I and II of the dorsal horn, where they show a complete overlapping (Fig. 4.2 A-C). In the caudal SG, CGRP- and SP-ir neurons show the same brilliant fluorescence (Fig 4.2 D-F). CGRP-ir neurons co-expressing SP are 45 ± 11% (188/410 cells, $n = 3$) whereas caudal SP-ir neurons co-expressing CGRP are 57 ± 8 % (188/318 cells, $n = 3$). Unfortunately, IB4-binding was not evaluable in all animals, due to its frequently weak signal. Although we are not able to quantify IB4-labelled neurons of the bottlenose dolphin SG, we would like to report that IB4 results co-localized with CGRP and/or SP in primary

afferent neurons (Fig. 4.4) as in the first laminae of the spinal cord (Fig. 4.3). In primary sensory neurons, IB4-labelling was associated with plasmatic granules (Fig. 4.4 E, H).

Discussion

To the best of authors' knowledge, this is the first description of CGRP-ir neurons and fibres in the spinal cord and spinal ganglia of a marine mammal, including cetacean species. Just like in other animals, also in the bottlenose dolphin the majority of CGRP-ir fibres are localized in the superficial laminae of Rexed (i.e. I and II) throughout the whole spinal cord. These fibres convey information coming from primary afferent neurons of SG. In other mammals, numerous CGRP-ir fibres have been described in lamina V, some of them running until the dorsal extension of lamina X. A lesser density of ir-fibres has been observed connecting lamina II and lamina X and around the central canal. (Gibson et al., 1984; Conrath et al., 1989; Chang et al., 2008). In the bottlenose dolphin we observed only few ir-fibres in the lamina X while there seems to be a completely lack of stained neuronal cell bodies in lamina V. Neurons of lamina V, which have their dendrites in lamina II, receive nociceptive information from visceral organs mainly. Therefore, in the dolphin they might not be involved in the transmission of pain. As demonstrated in other species, it is possible to speculate that also in the bottlenose dolphin the great majority of immunostained fibres of the dorsal horn are of extrinsic origin, probably derived from neurons of the SG. In the dorsal horn, the interaction between descending modulatory pathways, inhibitory and excitatory interneurons and projection neurons influences the activity of these latter cells. Although the superficial laminae of the spinal cord have been deeply investigated, it has to be said that many aspects of their organization and neuronal connections are still unknown. In the first two laminae of the dolphin spinal cord, few small neurons show an intense CGRP-immunoreactivity. Most of the small neurons of lamina II and a great part of neurons of laminae I and III are considered to be interneurons (Todd, 2010). As it is difficult to discriminate between interneurons and secondary afferent neurons only based on their morphology or limited neurotransmitter phenotype, we can only speculate on the

function of these few CGRP-ir neurons and we cannot exclude that they actually represent projection neurons. The presence of CGRP-immunoreactivity in the motor neurons of the ventral horn (lamina IX) have already reported in other species (Gibson et al., 1984; Chang et al., 2008). The peptide synthetized in these cells is then displaced to the neuromuscular junction where it is stored with acetylcholine and released upon nerve stimulation (Matteoli et al., 1988; Kashihara et al., 1989; Uchida et al., 1990; Van Rossum et al., 1997). In the thoracic tract of dolphins, CGRP-immunoreactivity has been also found in neurons of the intermediolateral column, which are known to be motor neurons that give rise to preganglionic sympathetic visceral efferents.

Primary afferent neurons can be classified according to different and interrelated features such as conduction velocity, neurochemical phenotype, peripheral targets and size. The literature reports several classifications of SG neurons based on this latter parameter. The most common subdivision considers three classes: small, medium and large size neurons. Small (C-type) neurons have unmyelinated axons and the slowest conduction velocity. Medium (Aδ) and large (Aβ) neurons have lightly and heavily myelinated processes, and consequently intermediate and rapid conduction velocities, respectively (Marmigere and Ernfors, 2007). Based on responses to different kind of noxious stimuli, the majority of Aδ and C fibers are thought to be nociceptive or thermoceptive, while Aβ afferents are low-threshold mechanoceptors (Almeida et al., 2004; Todd, 2010; Abraira and Ginty, 2013). There are several differences regarding the limits defining each size category and the great majority of studies concerns small laboratory animals. In our previous research on dolphins SG (Bombardi et al., 2010, 2011), we decided to apply the same method applied by Lukácová et al. (2006) in the dog and Russo et al. (2010) in the sheep, considering neurons with an area up to 1000 μm^2 to be small, neurons between 1000 and 2000 μm^2 to be medium, and neurons over 2000 μm^2 to be large. However, at this time, a first rough evaluation of data obtained made us hypothesized that this was not the best fit for our data. In fact, in Nissl preparations, some neurons reach an area of approximately 7000μm^2. Through statistical analysis, we could identify four classes of primary sensory neurons in the caudal spinal ganglia of the bottlenose dolphin with most

of the CGRP-ir cells belonging to small and medium-size groups. Based on their neurochemical profile, the subpopulation of SG nociceptors has been classically divided in two groups, peptidergic (showing CGRP and SP immunoreactivity) and non-peptidergic (showing IB-4 labelling). Various studies in the mouse support the assumption that peptidergic and IB4-binding nociceptors are separate entities (Zwick et al.,2002; Woodbury et al., 2004). On the contrary, a co-expression of IB4 with CGRP and/or SP has been demonstrated in other animals as rat (Kashiba et al., 2001; Price and Flores 2007), sheep, (Russo et al., 2010), horse (Russo et al., 2011) and swine (Russo et al., 2013). Due to the weak labelling of IB4 we decided not to quantify IB4+ neurons in the SG of the bottlenose dolphin. Nevertheless, it has to be said that we observed a certain degree of co-localization with both CGRP and SP. Some authors (Silvermann and Kruger, 1990; Wang et al., 1994) report that peptidergic and non-peptidergic neurons seem to differ also in their central projection, as peptidergic fibres are reported to terminate mainly in lamina I and outer lamina II, while non-peptidergic fibres in inner lamina II. However, a more recent study reported an overlap of CGRP, SP and IB4 terminations in the dorsal horn (Gerke and Plenderleith, 2002), just like what we observed in the spinal cord of the bottlenose dolphin. In primary afferent neurons as in motor neurons of the ventral horns, we observed that IB4 labelling was associated with cytoplasmic granules, mostly arranged around the nucleus. Ultrastructural studies carried out in the rat reported that these granules correspond to the Golgi apparatus (Streit et al., 1986; Gerke and Plenderleith, 2002).

In conclusion, the CGRP-immunoreactivity expression pattern in the spinal cord and SG of the bottlenose dolphin matches what described in the literature for most mammalian species (Gibson et al., 1984; Skofitsch and Jacobowitz 1985; Hökfelt et al., 1992; Batten et al., 1989; McNeill et al., 1988; Conrath et al., 1989; Van Rossum et al., 1997). The percentage of CGRP-ir primary sensory neurons in the caudal ganglia of the bottlenose dolphin results quite similar to values obtained in the thoracic and lumbar tracts of horse and sheep (Russo et al., 2010, 2011). The large presence of CGRP in sensory and motor system of the bottlenose dolphin is suggestive of a heterogeneous

functional picture for this peptide. As reported for other animals, also in the bottlenose dolphin, the isolectin-B4 is not a suitable marker for classifying small and medium size sensory neurons in peptidergic and non-peptidergic subpopulations.

Tab 4.1. Number of individuals for each tract

tract (SC + SG)	n
CERVCIAL	2
TORACIC	3
LUMBAR	2
CAUDAL	3

Abbreviations: SC Spinal cord; SG spinal ganglia.

Tab 4.2. Details of the antibodies and NeuroTrace® used

Primary Antibodies and NT	***Host***	***Code***	***Dilution***	***Source***
CGRP	rabbit	C8189	1:1000	Sigma-Aldrich
SP	rat	10-S15A	1:300	Fitzgerald
IB4 biotin conjugate		L2140	1:100	Sigma-Aldrich
NT			1:200	Molecular Probes
Secondary Antibodies			***Dilution***	***Source***
biotinylated anti-rabbit (BA1000)			1:200	Vector
Goat anti-rabbit IgG FITC (401314)			1:200	Calbiochem Novabiochem
Donkey anti-rat IgG Alexa 594 (A21209)			1:50	Invitrogen
Goat anti-rat 488 (20023)			1:80	Biotium
Streptavidin Texas Red-conjugated (SA-5006)			1:200	Vector
Streptavidin AMCA-conjugated (SA-5008)			1:200	Vector

Abbreviations: CGRP calcitonin gene-related peptide, SP substance P, IB4 isolectin B4 from *Griffonia simplicifolia*, NT blue fluorescent Nissl stain solution, FITC fluorescein isothiocyanate, AMCA aminocoumarin.

Suppliers: Sigma-Aldrich, St. Louis, MO, USA. Fitzgerald Inc. Concord, MA, USA. Molecular Probes, Eugene, OR, USA. Vector, Burlingame, CA, USA. Calbiochem Novabiochem, San Diego, CA, USA. Invitrogen,Therno Fisher Scientific, Rockford, IL, USA. Biotium, Fremont, CA, USA.

Figures

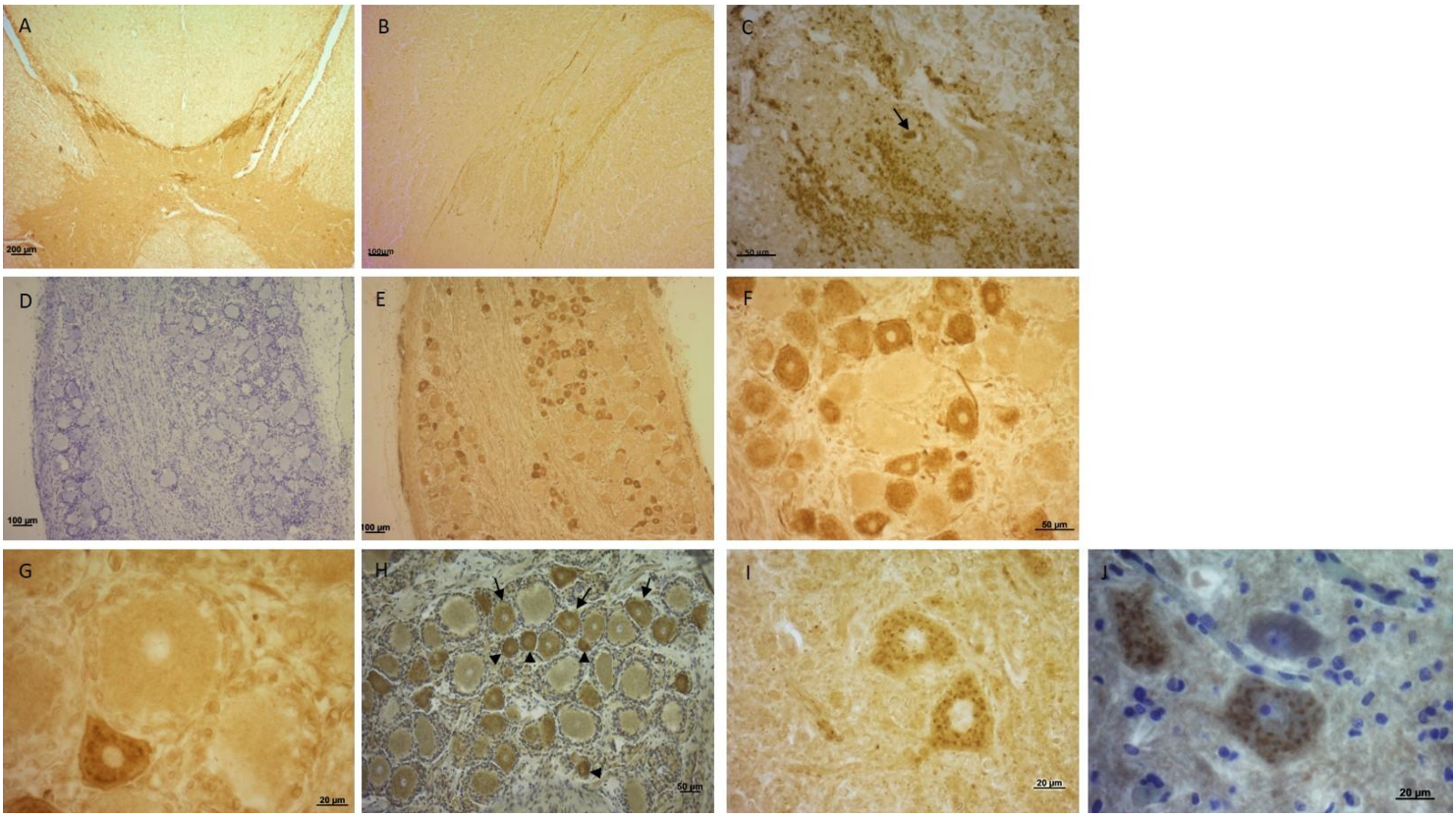

Fig. 4.1. **A-C**) Micrographs of caudal spinal cord showing CGRP-immunoreacive (IR) fibers and varicosities in laminae I, II, X of the dorsal horn (**A**) and Lissauer's tract (**B**). In the superficial laminae of the dorsal horn few small cells show an intense labelling (arrows) (**C**). **D-G**) Nissl staining (**D**) and immunoperoxidase tecnique showing CGRP immunoreactivity (**E,F,G**) in a caudal spinal ganglion. **H**) Counterstained section of caudal spinal ganglia showing small (arrowheads) and medium-size (arrows) immunoreactive primary afferent neurons (asterisks). **I-J**) Motor neurons of lamina IX showing the typical granular aspect of CGRP-immunoreactivity.

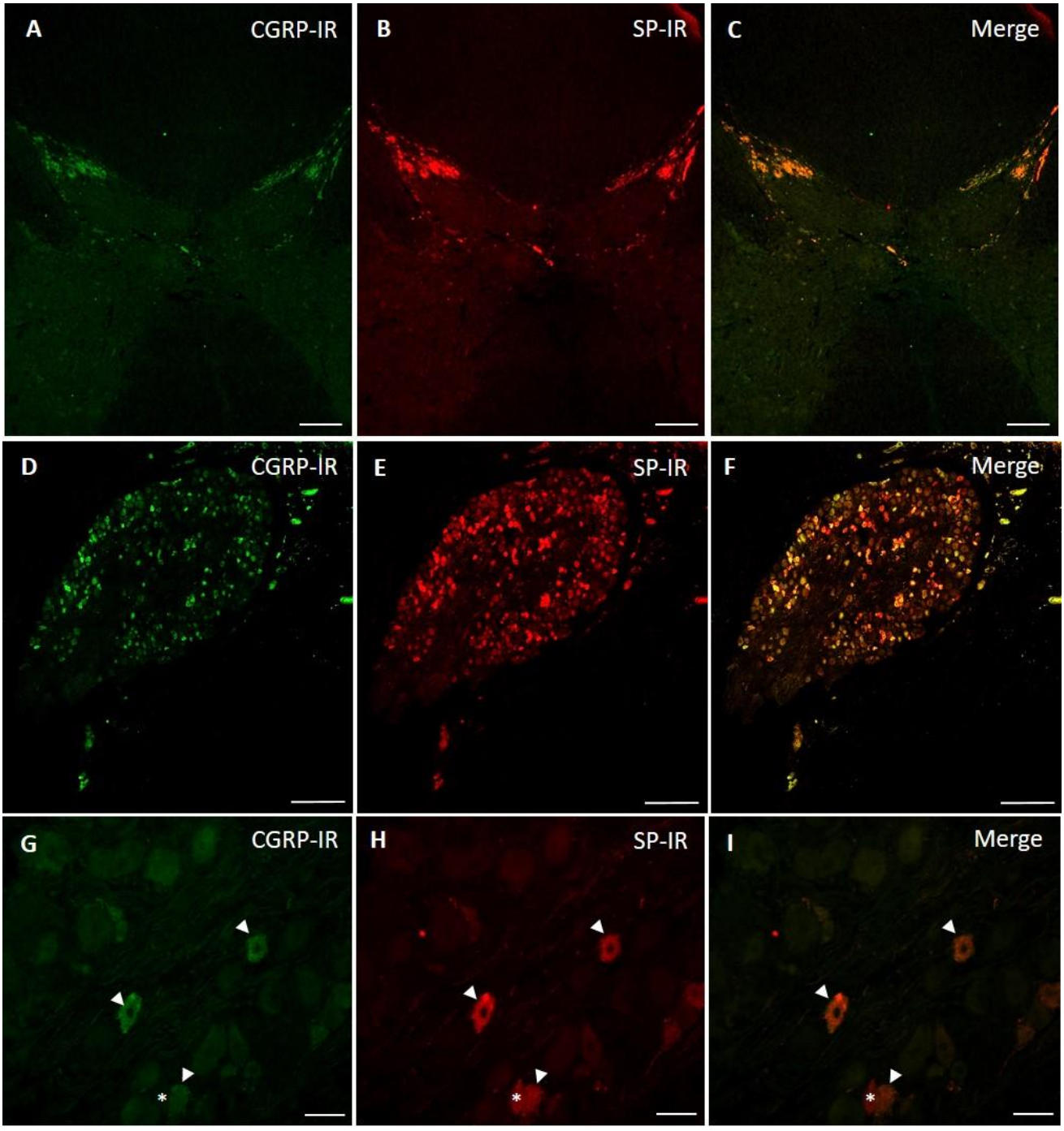

Fig. 4.2. A-F) Micrographs showing co-localization between CGRP and SP in fibers and varicosities of the dolphin dorsal horn (**A-C**), and in primary sensory neurons of a caudal spinal ganglion (**D-F**). **G-I**) Primary afferent neurons co-expressing CGRP and SP (arrowheads). Asterisk indicate a neuron SP-immunoreactive but negative for CGRP. A-C) scale bar 200 µm. D-F) scale bar 500 µm. G-I) scale bar 20 µm

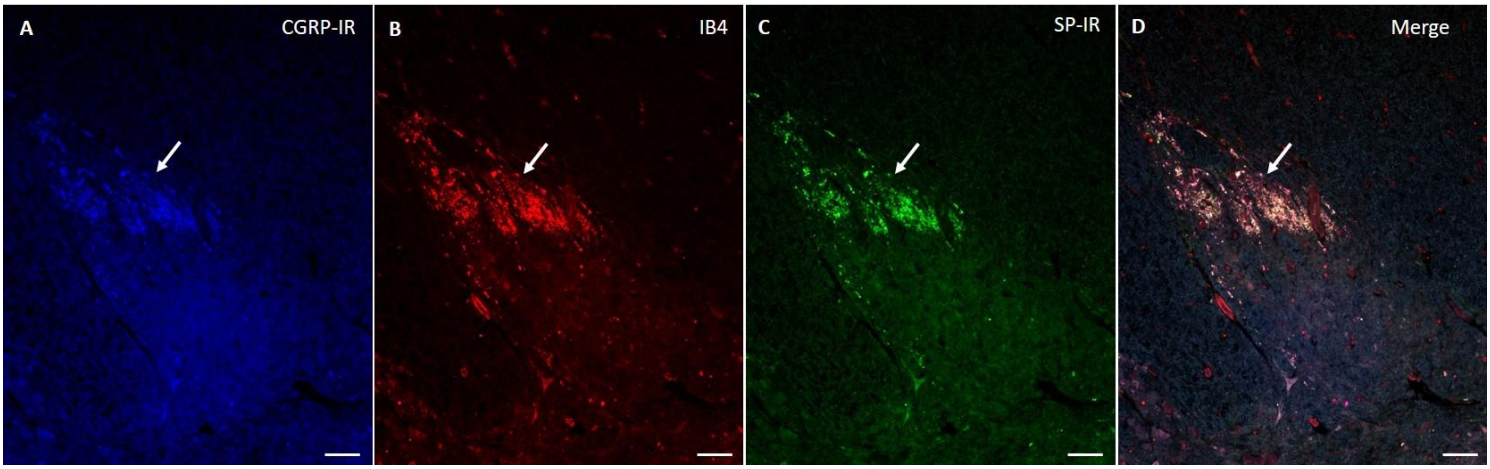

Fig. 4.3. A-D) CGRP/IB4/SP triple staining in caudal sections of bottlenose dolphin spinal cord. Arrow shows fibers and varicosities in the firts laminae of the dorsal horn immunoreactive for both CGRP (**A**) and SP (**C**) and IB4- labelled (**B**). Scale bar 50 µm.

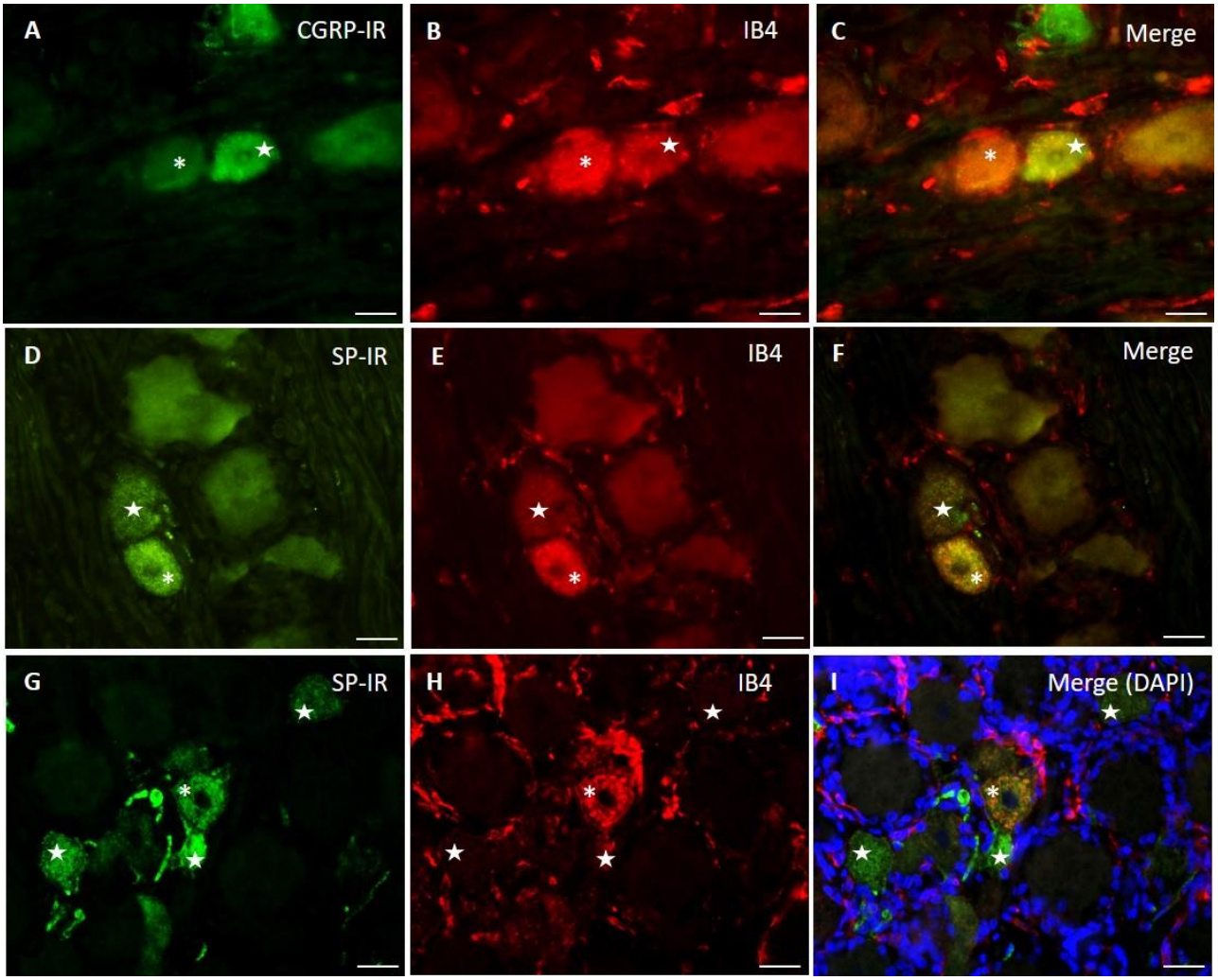

Fig. 4.4. Micrographs of dolphin SG sections. A-C) White star shows a calcitonine gene-related peptide (CGRP) immunoreactive (-IR) neuron negative for IB4-labelling. Asterisk indicates CGRP-negative and IB4 labelled neuron. **D-F**) Asterisk indicates SP-IR and IB4-labelled neuron while white star show a SP-IR neuron, which was IB-4 negative. **G-I**) White stars indicate some SP-IR neurons, which were negative for IB4-labelling while asterisk shows a neurons SP-IR and IB4-labelled. Note that the IB4-labelling is associated with plasmatic granules. A-I) Scale bar 20 µm

CHAPTER 5

Nitrergic and substance P immunoreactive neurons in the enteric nervous system of the bottlenose dolphin (*Tursiops truncatus*) intestine

Introduction

Marine Cetartiodactyla, including the bottlenose dolphin (*Tursiops truncatus*), underwent profound morphological and physiological evolutionary adaptations to life in the water (Reidenberg, 2007). Salinity and wide variations of temperature and pressure are just a few of the environmental characteristics that cetaceans have to deal with. As other organs and systems of odontocetes, also the gastrointestinal (GI) tract presents structural and functional differences with terrestrial mammals. The tongue shows no taste buds except in selected species (Cozzi et al., 2017), preys are grabbed and swallowed whole without being chewed; the larynx the can be voluntarily displaced to allow passage of food passes through the pharynx (Reidenberg and Laitman 1987; Cozzi et al., 2017). The stomach of delphinids consists of multiple chambers, including a highly muscular forestomach, necessary to grind and digest the whole prey (Harrison et al, 1970; Gaskin, 1978; Mead, 2008). Although multiple chamber-stomachs are also characteristics of several terrestrial Cetartiodactyla (including the closely related ruminants and *Hippopotamidae*), the stomach complex of cetaceans does not promote multiple chewing cycles. The intestine does not show any macroscopical subdivision and lacks the caecum; also the histological aspect of the intestine results rather constant, without remarkable differences between the anterior and posterior part (Gaskin., 1978; Mead, 2008; Russo et al., 2012). Cetaceans also lack a gall bladder, a consequence to the continuous ingestion of food (Mead, 2008).

Although the digestive system of cetaceans presents such differences in comparison with terrestrial mammals, still few information is currently available on their enteric nervous system (ENS) (Pfeiffer, 1993; Domeneghini et al., 1997; Naka et al., 2007; Russo et al., 2012; Gatta et al., 2014). The ENS regulates the great majority of digestive functions and activities such as motility, absorption, secretion and blood flow. It consists of a huge integrated net of neurons and fibres arranged in the wall of the digestive system, from the oesophagus to the internal anal sphincter, extending also to the pancreas and extrahepatic biliary system. (Grundy and Schemann, 2005; Furness, 2006). In the ENS, neurons, fibres and enteric glial cells are organized in two major

ganglionated plexuses, the myenteric (MP) and submucosal plexus (SMP). The MP is located between the longitudinal (LML) and circular (CML) muscle layers, and provides motor innervation to the GI smooth muscle cells, while the SMP regulates mainly mucosal and submucosal functions and activities, at least in small laboratory rodents (Furness, 2006). Enteric neurons can be grouped in different functional classes (intrinsic primary afferent neurons –IPANs -, excitatory and inhibitory motor neurons, and interneurons), characterized by their neurochemical code (i.e. the cocktail of neurotransmitters that they synthesize). In the GI tract, the most important inhibitory neurotransmitter is represented by the nitric oxide (NO). Mostly released by myenteric neurons, NO is able to induce relaxation of the GI musculatures and sphincters, by directly acting on the intestinal smooth muscle cells or by attenuating the release of the excitatory neurotransmitters, such as acetylcholine and substance P (SP) (Sanders and Ward, 1992; Stark et al, 1993). NO is synthesized through the activation of neuronal nitric oxide synthase (nNOS), an enzyme that can be found in MP and SMP neurons and fibres of different species (Costa et al., 1992; Ekblad et al, 1994; Timmermans et al., 1994a,b). SP belongs to the tachykinins family, a group of neuropeptides involved not only in the regulation of different gastrointestinal functions, such as motility and secretion, but also in the inflammation and pain genesis (Holzer and Holzer-Petsche, 1997a,b; Maggi et al, 1997; Shimizu et al., 2008; Steinhoff et al., 2014). In the gut, SP is found in excitatory muscular motor neurons and submucosal IPANs, as well as in extrinsic sensory fibres and enteroendocrine cells (Furness, 2006; Domeneghini et al., 2007; Shimizu et al., 2008). Frequently detected with acetylcholine in intestinal intramural neurons and fibres, SP is considered a cholinergic co-mediator (Brookes, 2001; Furness, 2006). Since nNOS and SP (and its receptors) play important roles in the intestinal motor function, they have been widely considered as relevant neurotransmitters in the study of GI motility disorders (Sivarao et al., 2008; King et al., 2010; Cellini et al., 2012; Masaoka et al, 2014). Notably, a recent research hypothesized the employment of dolphins as a natural animal model in studying diabetes (Venn-Watson et al., 2011). In fact, bottlenose dolphins seem to be able to switch in and out a condition of metabolic changes similar to

those found in diabetic human patients. When fed a high protein fish meal, dolphins mimic a type 2 diabetes-like state (sustained postprandial hyperglycaemia and hyperinsulinemia), while they mimic a type 1 diabetes-like state (sustained postprandial hyperglycaemia but insulin deficient response) after oral dextrose administration (Venn-Watson and Ridgway, 2007; Venn-Watson et al., 2011, 2013; Venn-Watson, 2014). It is well assumed that diabetes mellitus in humans is associated with GI dysfunctions, affecting both the upper and the lower tract, including the stomach and the small and large intestine (Maleki et al., 2000; Bytzer et al., 2001; Sellin and Chang, 2008; Ordog et al., 2009). As demonstrated by several studies, carried out both on diabetic patients and induced diabetes models in mice and rats, enteric nitrergic neurons show great susceptibility to diabetes, especially in the early phases of the disease (Wrzos et al., 1997; Watkins et al., 2000; Cellek et al., 2003; Chandrasekharan and Srinivasan, 2007; Chandrasekharan et al., 2011; Rivera et al., 2011; Yarandi and Srinivasan, 2014). Loss of nNOS-immunoreactive (IR) neurons was observed in MP of human patients affected with both type 1 and type 2 diabetes (He et al., 2001; Iwasaki et al., 2006). Here we also note that the gut is a major extrapineal site of melatonin synthesis in bottlenose dolphins, thus suggesting an important role for the intestine in the regulation of circadian and circannual rhythms (for review and discussion see Panin et al., 2012).

The aim of the present study was to immunohistochemically investigate morphological and quantitative aspects of nNOS- and SP-IR enteric neurons in non-pathological gut of bottlenose dolphins. This way we provide a first insight on the complex interaction between two major neurochemical classes of enteric neurons in the intestine of a very common marine mammal.

Materials and methods

Samples of intestine of three adult bottlenose dolphins were obtained from the *Mediterranean marine mammal tissue bank* of the Department of Comparative Biomedicine and Food Science of the University of Padova (Italy) (MMMTB, www.marinemammals.eu). The MMMTB (CITES IT020) works under the auspices of the Italian Ministry for the Environment and

the University of Padova, and receives tissue specimens from cetaceans stranded along the Italian coasts of the Mediterranean, or directly samples tissues from dolphins and whales brought to its facilities for post-mortem diagnosis. All carcasses are coded for freshness. Once removed, tissue fragments were washed in PBS (0.1 M phosphate buffer saline, pH 7.4) and immersed in 4% buffered formalin for at least 24 h at 4 °C; following fixation, tissue samples were dehydrated and embedded in paraffin. Serial longitudinal and transverse sections (7 µm thick) were collected on poly-L-lysine–coated slides and processed for histological and immunohistochemical labelling.

Histology

One section for each specimen was stained with haematoxylin and eosin (H&E) to assess tissue condition. Microscopic analysis of the sections showed absence of pathological alteration in the gut, and therefore tissues from all the three animals were included in the present research.

Double Immunofluorescence

Sections were deparaffinized in xylene, rehydrated through graded ethanols and heated in sodium citrate buffer (pH 6.0) in a microwave (5 min at 700 W) for antigen retrieval. To block non-specific bindings, sections were incubated for 1,5 h at room temperature (RT) in a solution containing 20% normal goat serum (CS9022, Colorado Serum Co., Denver, CO, USA), 20% normal donkey serum (D9663, Sigma-Aldrich, Saint Louis, MO, USA), and 0.5% Triton X-100 (Merck, Darmstadt, Germany) in PBS. Sections were then incubated overnight in a humid chamber at RT in a cocktail of primary antibodies (Table1) diluted in 1.8% NaCl in 0.01M PBS containing 0.1% sodium azide. After rinsing in PBS (3 x 10 min), the sections were incubated for 1,5 h at RT in a solution of secondary antibodies (Table 1) diluted in PBS. Enteric neurons were identified using the blue fluorescent Nissl stain solution (NeuroTrace®, Molecular Probes, Eugene, OR, USA – NT through the text). After washing, the sections were mounted in buffered glycerol at pH 8.6. Secondary antibody specificity was tested by omitting primary antisera.

Analysis of Sections

Immunohistochemical preparations were analysed with a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes. The images were recorded with a DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). The proportions of neurons that were NT-labelled and that were immunoreactive for nNOS or SP were determined by examining fluorescent double-stained preparations. Neurons were first located by the presence of a fluorophore that labelled NT and then the filter was switched to determine whether or not the neuron was labelled for a second antigen (nNOS or SP), located with a fluorophore of a different colour. In this way, proportions of neurons labelled for pairs of antigens were determined. At least 200 NT-labelled neurons were counted for each sample from each animal and the percentage of neurons that were NT-labelled and that were also immunoreactive for nNOS or SP was calculated and expressed as relative percentage (median and interquartile range –IQR).

Results

Histologic study

In the bottlenose dolphin, the MP was organized in large ganglia located between the longitudinal (LML) and circular muscle layer (CML). In longitudinal and transverse sections, ganglia showed different size and contained up to 43 neurons. Neurons of the SMP were organized in smaller ganglia (up to 18 neurons) distributed at two different levels within the submucosal layer. The inner submucosal plexus (ISMP) was composed of small ganglia located near the *muscularis mucosae* harboring small somata, whereas the outer submucosal plexus (OSMP), lying close to the CML, was composed of larger neurons. Solitary neurons were also observed, dispersed in the submucosal layer.

Nitrergic neurons

The nitrergic subpopulation of enteric neurons presented homogeneous immunoreactivity of the soma without nuclear labelling. These neurons showed irregular outline and short processes, resembling Dogiel type I neurons. In the MP, nNOS-IR neurons represented 28 % (IQR = 19-29) of the total neuronal population (404/1478 cells; *n* = 3) (Fig. 5.1). In the SMP, nNOS-IR neurons were observed just occasionally (1% IQR = 0-2), and only in the OSMP, close to the CML (9/1132 cells, n = 3) (Fig. 5.2).

SP-IR neurons

The SP-IR was expressed by 31 % (IQR= 22-37) of MP (456/1478 cells, n = 3) and 41% (IQR= 24 -63) of SMP (412/1132cells, n = 3) neurons. The majority of SP-IR myenteric neurons showed a smooth outline of the cell body, typical feature of the Dogiel type II neurons (Fig. 5.1; Fig. 5.2). In the myenteric neuropil, we frequently observed bright SP-IR nerve fibres forming baskets of SP-IR varicosities around nNOS-IR and nNOS-negative neurons. Frequently, immunolabelled varicosities and fibres were visible around submucosal blood vessels (data not shown).

Co-localizations of nNOS- and SP

Any co-localization between the two markers was detected, in neither the plexuses.

Discussion

Our observations on the histological architecture of ENS in the intestine of the bottlenose dolphin confirm what previously described by other authors (Gaskin, 1978; Pfeiffer, 1993; Domeneghini et al., 1997; Russo et al., 2012). A multi-layered distribution of the submucosal ganglia was already reported in the intestine of other large mammals (Schabadasch, 1930; Gunn, 1968; Stach, 1977; Christensen and Rick, 1987; Scheuermann et al., 1987a, b; Timmermans et al., 1992, 1997, 2001; Pearson, 1994; Pompolo, 1994; Balemba et al., 1998, 1999). Several

neurotransmitters, including tachykinins and NO, provide key regulation of the contractility of the GI musculature. The balance between excitatory and inhibitory signals to smooth muscle cells generates all the intestinal physiological motor patterns. As NO is the main transmitter involved in inhibitory inputs and SP is considered a co-transmitter in cholinergic neurons, the present study provides a first comprehensive insight on these two functional classes of neurons in the intestine of the bottlenose dolphin. Our data on nitrergic MP subpopulation (28%) are generally consistent with those obtained in other large (Brehmer et al, 2004; Chiocchetti et al., 2006, 2009; Mazzuoli et al., 2007; Freytag, 2008; Zacharko-Siembida et al., 2013; Giancola et al 2016) and small (Furness 2006; Qu et al., 2008; Lawson et al., 2010) terrestrial mammals, despite some differences regarding the intestinal tract considered. The percentage obtained in the MP of bottlenose dolphins diverges just slightly from data obtained in the human small and large intestine, where nitrergic neurons represent about 34% and 38%, respectively (Brehmer et al., 2006). In the SMP, the nNOS-IR was expressed only by 1% of neurons, exclusively located in the large ganglia close to the CML, whereas they were absent in the ISMP. These percentages are very similar to those previously described in the horse ileum (Chiocchetti et al., 2009) and in the small intestine of piglets (Zacharko-Siembida et al., 2013), where nNOS-IR neuronal cells represented respectively about 5% and about 1% of the total SMP neurons and were generally located in the OSMP. On the other hand, our percentages are quite far from those obtained in the lamb (Chiocchetti el al., 2006) and persian squirrel ileum (Sadeghinezhad et al., 2013), where SMP nNOS-IR neurons represent 21% and 11%, respectively. The great majority of nNOS-IR cells observed in the present research were Dogiel type I shaped. Dogiel type I cells, characterized by an angular outline of the soma and numerous dendritic processes, include interneurons and inhibitory motor neurons (Furness, 2006). Interestingly in the bottlenose dolphin intestine, we observed the presence of nitrergic neurons in the OSMP but not in the ISMP. In other species it is well assumed that the ISMP and the OSMP present different neurochemical coding and different electrophysiological properties and therefore distinct functions (Timmermans et al., 1990, 2001; Crowe et al., 1992; Thomsen et al., 1997a,b). As

OSMP neurons actually show a phenotype more similar to that of the myenteric plexus neurons, it is plausible to think that they supply an inhibitory innervation of the CML (Scheurmann et al. 1988; Hens et al., 2000). Several pathological conditions of the gastrointestinal tract of human and other mammals are associated with an impairment of NO neurons (Vanderwinden et al., 1993; Bealer at al., 1994; Tomita et al., 1995; Takahashi et al., 1997; Ribeiro et al., 1998; Spangeus et al., 2000; Takahashi, 2003; Sivarao et al., 2008; Rivera et al., 2011; Masaoka et al, 2014; Giancola et al., 2016). Thus, to better understand the implications of nitrergic neurons in gastrointestinal pathologies of dolphins is essential to investigate chiefly the architecture and neurochemistry of the ENS, particularly in its inhibitory components, in tissues of healthy animals. To our knowledge, the distribution of SP-IR in the gastrointestinal tract of cetaceans was described only in the striped dolphin (*Stenella coeruleoalba*) by Domeneghini et al. (1997), who reported generic values (i.e. small, medium or high number) of intestinal SP-IR structures. In the bottlenose dolphin intestine myenteric SP-IR subpopulation accounts for 31% of the total neuronal population. This value results quite higher compared to the percentages reported in the ileum of horse (14%) (Chiocchetti et al., 2009), sheep (13%) (Mazzuoli et al., 2007) and dog (15%) (personal observations of Dr. R. Chiocchetti), and in the small intestine of suckling pigs (~1%) (Zacharko-Siembida et al., 2013). In the SMP of the bottlenose dolphin SP-IR neurons represent 41% of the total neuronal population and where largely located in the ISMP. This percentage results similar to what described in the ileum of the sheep (38%) (Chiocchetti et al., 2006; Mazzuoli et al., 2007) and in the small intestine of piglets (from 33% to 42%, depending on the tract) (Zacharko-Siembida et al., 2013), whereas differs significantly from data obtained in the horse ileum (Chiocchetti et al., 2009) and in the colon of piglets (Petto et al., 2015), where SP-IR neurons achieve 66% and 87% of the total neuronal population, respectively. In the dog ileum only 26% of SMP neurons results SP-IR (personal observations of Dr. R. Chiocchetti). The majority of SP-IR neurons observed in the present study mostly resembles Dogiel type II cells. SP-IR neurons could possibly be IPANs, interneurons, or excitatory muscular motor neurons (Sang et al., 1997; Clerc et al., 1998; Furness, 2006). As

reported in other mammals like the striped dolphin (Domeneghini et al., 1997) and the horse (Chiocchetti et al., 2009), also in the bottlenose dolphin we observed baskets of SP-IR varicosities and fibres around the MP nitrergic and non-nitrergic neurons. These fibres, located around somata, might arise from IPANs and interneurons (Furness et al., 2004) or from peripheral processes of dorsal root ganglion afferent neurons which participate in the control of gastrointestinal activities (Holzer, 2007). Interesting findings in other marine Cetartiodactyla belonging to the *Ziphiidae* and *Delphinapteridae* families (Pfeiffer, 1993) reported a modified innervation of the myenteric plexus and changes in the *muscularis externa*. Specifically, the musculature of the gut showed the presence of intercalation-like striations, thus supporting the presence of voluntary (and involuntary) movements, presumably essential for the peculiar modality of suction-feeding (Pfeiffer, 1993; Cozzi et al., 2017). The presence and distribution of visceral nitrergic and substance P immunoreactive neurons that we report here may further support the existence of such physiological mechanisms.

Differently to what described in the sheep (Mazzuoli et al., 2007) and similarly to what described in the small intestine of the mouse (Qu et al., 2008), no co-localization between nNOS- and SP-IR was detected in either the plexuses, suggesting the existence of two completely distinct functional classes of neurons in the intestine of the bottlenose dolphin.

Conclusions

This is, to the best of Authors' knowledge, the first description and quantification of nNOS-IR neurons, and the first quantification of SP-IR neurons as well, in the intestine of a cetacean species.

Although the general characteristics and morphology of nNOS-and SP-IR neurons result maintained among mammals, we found some differences in the percentage of neurons expressing the two markers, consisting mainly in a very scarce number of nNOS-IR neurons in the SMP, and a higher number of MP SP-IR neurons, compared with other species. Further investigation is needed to

identify neurochemical classes of neurons and fibres in order to give a clearer and more comprehensive picture of the ENS complexity in this species. Providing information on the physiological conditions of a healthy intestine, even in its nervous component, is crucial to approach and understand each pathological states.

Tab 5.1. Details of antibodies and NeuroTrace® used

Primary Antibodies and NT	*Host*	*Code*	*Dilution*	*Source*
nNOS	rabbit	Ab 5380	1:300	Millipore
SP	rat	10-S15A	1:300	Fitzgerald
NT		N21479	1:200	Molecular Probes

Secondary Antibodies	*Dilution*	*Source*
Goat anti-rabbit IgG FITC	1:200	Calbiochem
Donkey anti-rat IgG Alexa 594	1:50	Invitrogen

Abbreviations: nNOS, neuronal nitric oxide synthase; NT blue fluorescent Nissl stain solution; SP, Substance P.

Figures

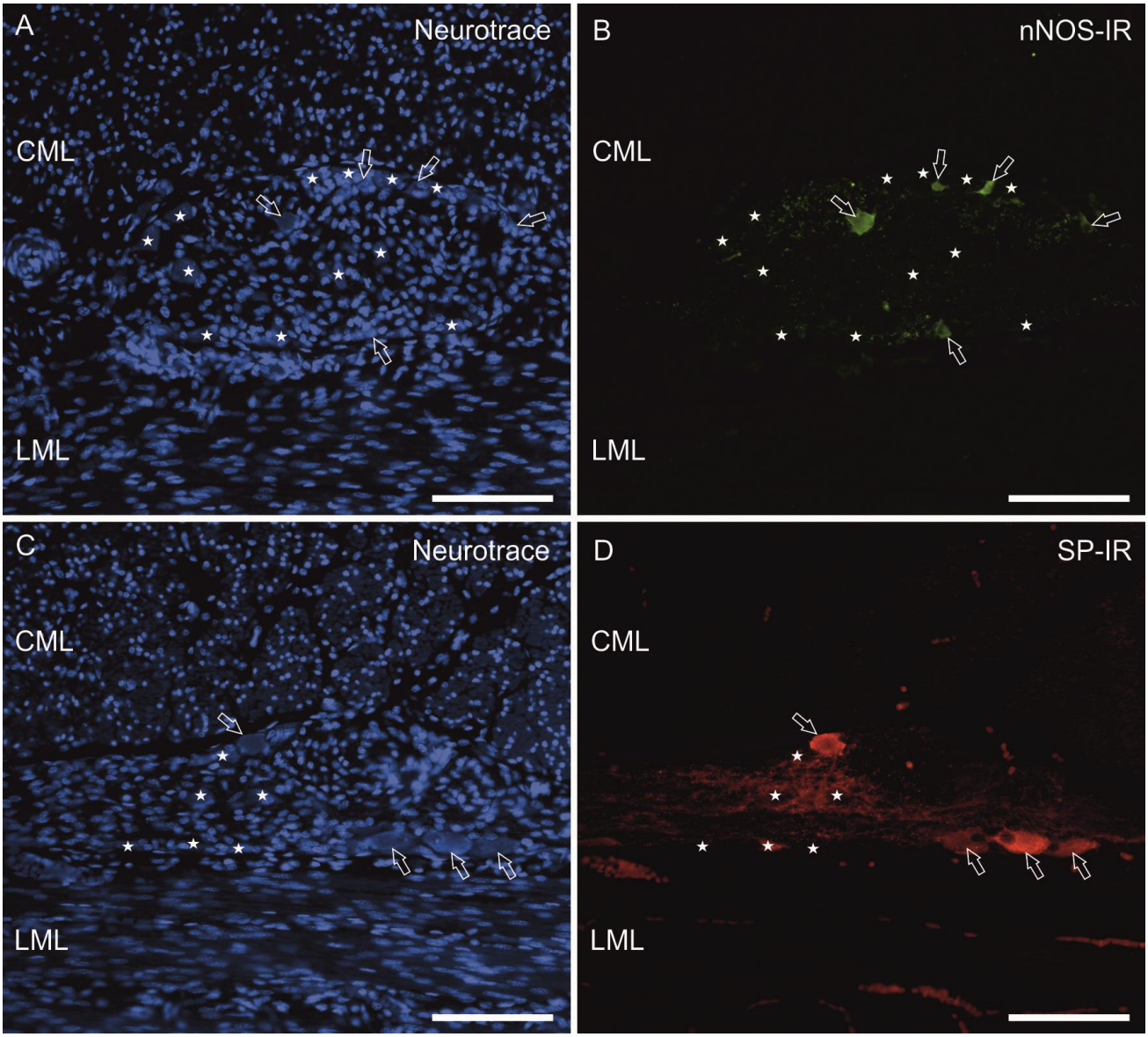

Fig.5 .1 Micrographs showing nNOS- (A-B) and SP-immunoreactivity (IR) (C-D) in longitudinal cryosections of the myenteric plexus in bottlenose dolphin intestine. White stars indicate myenteric plexus neurons labelled with the fluorescent tracer NeuroTrace (A, C) which were not immunoreactive for the neuronal nitric oxide synthase (nNOS-IR) and Substance P (SP-IR); open arrows indicate NeuroTrace-labelled neurons which were nNOS-IR (B) and SP-IR (D). Abbreviations: CML, circular muscle layer; LML, longitudinal muscle layer. Bars: a-d = 100 µm.

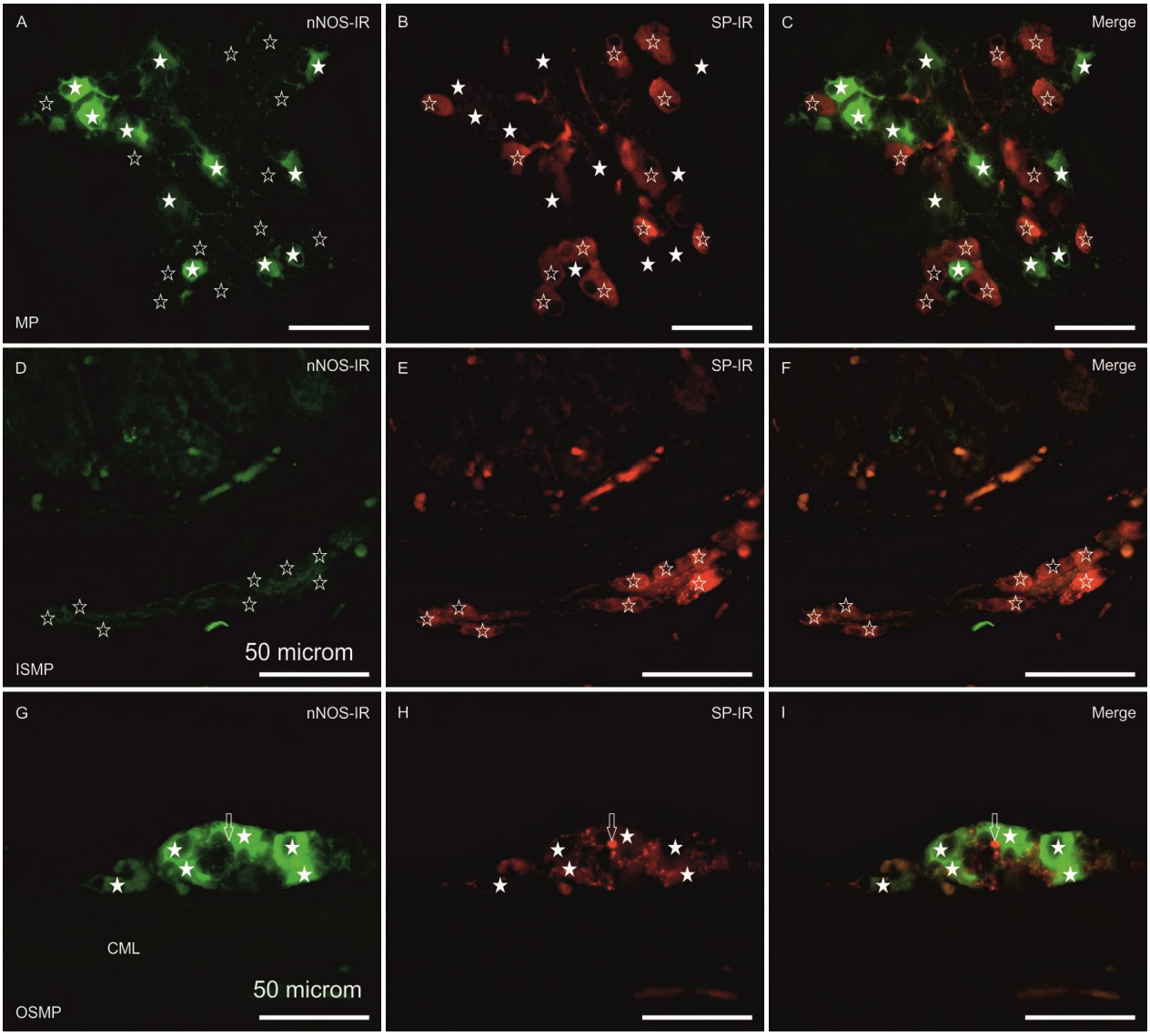

Fig. 5.2 Micrographs of cryosections of myenteric plexus (MP) (A-C) and submucosal plexus (SMP) (D-I) of the bottlenose dolphin intestine. White stars indicate neurons showing neuronal nitric oxide synthase (nNOS) immunoreactivity (nNOS-IR); open stars indicate neurons showing Substance P-IR (SP-IR). A-C) Cryosections in which it is evident that no MP neurons co-expressed the two neuronal markers. D-I) The SMP of the dolphin intestine was double layered; in the inner SMP (ISMP), neurons preferentially expressed SP-IR and were nNOS-negative; nitrergic neurons were only observable in the outer SMP (OSMP), in particular in neurons facing the inner portion of the circular muscle layer (CML). The arrow indicates SP-IR varicosities encircling nitrergic neurons. Scale bar A-I 50 µm

CHAPTER 6

Preliminary study on the expression of calcium binding proteins and neuronal nitric oxide synthase (nNOS) in the cortex and cerebellum of striped dolphins (*Stenella Coeruleoalba*) with morbilliviral meningoencephalitis

Introduction

Nonsuppurative (NS) meningoencephalitis has been frequently reported in different species of pinnipeds (Duignan et al., 1993, 2014; Bodewes et al., 2013; Donahoe et al., 2014) and cetaceans (Kennedy et al., 1992; Esperón et al., 2008; Di Guardo et al., 2010; Davison et al., 2013; Sierra et al., 2014) worldwide. In cetaceans, NS meningoencephalitis recognizes different causal agents like Morbillivirus, Herpesvirus, *Brucella ceti* and *Toxoplasma gondii*. Over the past 30 years Cetacean morbillivirus (CeMV) was proven to be involved in several mass mortalities of different cetaceans species (for review see Van Bressem et al., 2014), and the striped dolphin (*Stenella coeruleoalba*) seems to be the one of the most commonly affected species (van Bressem et al., 2009). CeMV (genus *Morbillivirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*) is a single-stranded RNA virus, which include three strains: the dolphin morbillivirus (DMV), the porpoise morbillivirus (PMV) and the pilot whale morbillivirus (PWMB) (Van Bressem et al., 2014). Recent studies reported the existence in the Pacific Ocean of a fourth strain affecting beaked whales (Jacob et al., 2016). Recently, DMV was proven to represent a potential threat also for other cetacean species like sperm whales and fin whales (Mazzariol et al., 2016, 2017). The literature reports three forms of disease caused by CeMV, an acute/subacute systemic form, a chronic systemic form and finally a chronic infection localized to the central nervous system (CNS). Lesions to the low respiratory tract predominantly characterize the acute/subacute systemic disease. Animals that survive the acute phase may die due to the immunosuppressive effect of the virus, which facilitates secondary infections. In both cases, typical morbillivirus lesions can be observed (i.e. necrotizing bronco-interstitial pneumonia, NS meningoencephalitis, and lymphoid depletion). Histologically, morbilliviral meningoencephalitis, as other NS meningoencephalitis, is characterized by mononuclear meningitis, gliosis, neuronal necrosis, neuronophagia, and mononuclear perivascular cuffs (Vandevelde et al., 2012). The chronic systemic form also is the consequence of secondary opportunistic infections but, in this case, histological findings in lung and brain are milder than in the acute/subacute form and frequently the viral antigen cannot be detected (Van Bressem et al.,

2014). The chronic nervous form share some histological features with the old dog encephalitis (Headley et al., 2009) and the human subacute sclerosing panencephalitis (Gutierrez et al., 2010). In this form, typical microscopic findings and viral antigen are exclusively detected in the nervous system (Van Bressem et al., 2014). This "only-brain" form has been increasingly reported among mediterranean (Di Guardo et al., 2011, 2013; Soto et al., 2011) and atlantic (Van Bressem et al., 2014) striped dolphins. Still little is known about neuropathogenesis and mechanisms underlying DMV neurovirulence. Neuronal damage during viral encephalomyelitis can result directly from viral infection or indirectly from the host immune response. Among suggested mechanisms for the neurodegeneration occurring in infectious and non-infectious encephalopathies, there is also excitotoxicity (Meldrum and Garthwaite, 1990). This is a pathological condition induced by an excessive release of glutamate, the major excitatory neurotransmitter in the adult mammalian CNS (Olney, 1969). This glutamatergic storm provokes an altered homeostasis of the intracellular ionic environment, mostly characterized by neural Ca^{2+} influx, which activates a catabolic process mediated by proteases, lipases, and nucleases, leading to neuronal damage and death (Sattler and Tymianski, 2000; Mark et al., 2001). Excitotoxicity was reported to be involved in several human neurodegenerative diseases (Dong et al., 2009), as well as in ischemic stroke (Hazell, 2007; Xing et al., 2012) and neuroinflammation caused by different viruses (e.g. Herpesvirus, West Nile virus, Alphavirus, Bornavirus, human coronavirus, japanese encephalitis virus, FIV) (Power et al., 1997; Darman et al., 2004; Nargi-Aizenman et al., 2004; Fotheringham et al., 2008; Blakely et al., 2009; Chen et al., 2012; Brison et al., 2014; Tizard et al., 2016). Using an in vitro model of canine distemper virus infection (which also belongs to the *Morbillivirus* genus), Brunner and colleagues (2007) described an increase in extracellular glutamate and a slowing down of neuronal loss after administration of glutamate receptors antagonists. Thus, they demonstrated the involvement of exocitotoxicity in the virus-induced cytopathic effect. Calretinin (CR) and calbindin-D28k (CB-D28k) are members of the superfamily of EF-hand calcium binding proteins (CaBP) and play a pivotal role as intracellular Ca^{2+} buffers (Baimbridge et al., 1992). An altered expression of these

proteins has been implicated in several neurological pathologies and their protective role against excitotoxic insults has been largely discussed (Scharfman and Schwartzkroin, 1989; Figueredo-Cardenas et al., 1998; Beers et al. 2001; D'Orlando et al., 2001, 2002; Eyles et al. 2002; Kuchukhidze et al., 2015). Furthermore, studying the distribution of CaBP in the nervous system allows one to identify specific neuronal chemotypes (DeFelipe, 1997). A limited number of studies has been published on the distribution of these proteins in the CNS of healthy cetaceans (Glezer at al., 1993; Hof et al., 1999; Hof and Sherwood, 2005; Kalinichenko and Pushchin, 2008; Cozzi et al., 2014) and, to the best of author's knowledge, no data on their expression under pathological conditions are available. Nitric oxide (NO) is an important gaseous molecule, which plays a broad range of physiologic functions in many organs and systems, like neurotransmission, neuromodulation, immune response, and regulation of blood flow (Guix et al., 2005; Hirst and Robson, 2011). NO is synthesized from L-arginine by enzymes known as nitric oxide synthases (NOSs). Three different isoforms of NOS have been identified, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Knowles and Moncada, 1994). While nNOS and eNOS are constitutive isoforms, active in neurons and endothelial cells, respectively, iNOS expression is activated in different cellular populations, like mononuclear immune cells, glial cells and endothelial cells, following pathogen recognition and cytokine release. Furthermore, while nNOS and eNOS activation is Ca^{2+}-dependent (requiring a transient calcium ions binding to calmodulin), iNOS exerts its activity in a Ca^{2+} independent manner. Another difference between the isoforms lies in their cellular localization: nNOS and iNOS are cytosolic whereas eNOS is associated to endothelial cells membrane (for review see Knott and Bossy-Wetzel, 2009). Although the nitrergic system is a dynamic entity pivotal for the CNS homeostasis, it can convert into a neurotoxic agent under pathological conditions. The potentially toxic effects are attributable to NO but also to its oxidation products (Lipton et al., 1993; Pacher et al., 2007). The involvement of NO and its toxic compounds in the pathogenesis of neurodegenerative diseases has been reported (for a general discussion see Guix et al., 2005). There is general agreement that an increase of iNOS is associated

to viral, bacterial and autoimmune meningoencephalitis (Koprowski et al., 1993; Zheng et al., 1993, Akaike et al., 1995), while data on nNOS are more controversial (Zheng et al., 1993; Akaike et al., 1995; Hooper et al., 1995; Shin et al., 2000). Nevertheless, a variation in the expression of constitutive nNOS, could be more suggestive of neuronal damage. Here we present a preliminary analysis on nNOS-, CR- and CB-D28k-immunoreactivity in different brain regions of striped dolphins with confirmed morbilliviral meningoencephalitis.

Materials and Methods

Seven striped dolphins (*Stenella coeruleoalba*) stranded along the coasts of the Canary Islands between 2001 and 2012 were included in the study. Four animals, IHC and PCR positive for DMV, exhibited various degrees of meningoencephalitic lesions (Tab. 6.1). The other three animals, included in the study as controls, showed no morphologic evidence of central neuroinflammation and died because of non-infectious causes (for details see Tab. 6.2). The animals with confirmed DMV infection were analyzed in a previous study (Sierra et al., 2014) in which the presence of other etiologic agents in the brain, which could have mimicked the same lesions (i.e. herpesvirus, *Toxoplasma gondii*, *Brucella ceti* and WestNile virus), was ruled out through PCR analysis. Immunohistochemistry was performed on formalin fixed, paraffin embedded samples of cortex and cerebellum. 4 µm thick sections were collected on Vectabond (Vector Laboratories, USA) coated slides and processed for immunohistochemistry. Sections were deparaffinized in xylene, rehydrated through graded ethanols and heated in sodium citrate buffer (pH 6.0) 10 min at 90-95° C for antigen retrieval. To block non-specific bindings, sections were incubated for 1 h at room temperature (RT) in a solution containing 10% normal goat serum (CS9022, Colorado Serum Co., Denver, CO, USA) in PBS. Sections were then incubated overnight in a humid chamber at 4° C in the primary antiserum (Tab. 6.3) diluted in 1.8% NaCl in 0.01M PBS containing 0.1% sodium azide. After rinsing in PBS (3 x 10 min), the sections were incubated for 1,5 h at RT in secondary antibody (Tab. 6.3) diluted in PBS. After rinsing in PBS, the sections were transferred to avidin–

biotin complex (ABC kit Vectastain, PK-6100, Vector Laboratories, Burlingame, CA) for 45 minutes at RT, and the immunoperoxidase reaction was developed by 3,30-diaminobenzidine (DAB kit, SK-4100, Vector Laboratories, Burlingame, CA). Sections were then rinsed, dehydrated in ethanol, cleared in xylene, and coverslipped with Entellan (Merck, Darmstaldt, Germany). To make easier the identification of the cytoarchitecture of the analysed regions and identify the cortical layers, sections adjacent to immunoperoxidase preparations were stained with 0.125% cresyl-violet solution for 10 minutes, dehydrated and coverslipped with Entellan (Merck, Darmstaldt, Germany). Data concerning the percentage of the image covered by immunoreactivity were obtained from five, randomly chosen, 10x sections, and analysed using the automatic threshold algorithm of ImageJ (http://rsb.info.nih.gov/ij/). Statistical analysis was performed and Mann-Whitney U test was used to compare mean values obtained in the "affected" and "control" groups in order to determine whether there was a significantly difference.

Results

Histological findings in the affected animals are reported in Tab. 6.1 and showed in Fig 6.2. As described in literature, Cetacean neocortex showed a general loss of granularization and by the lack of a clear layer IV. Another remarkable difference was the great thickness of layer I, compared to other mammals. Distribution of CR and CB-D28k-immunoreactivity in the cortex and cerebellum of the striped dolphins matched what previously described in other healthy cetaceans species (Glezer at al., 1993; Hof et al., 1999; Hof and Sherwood, 2005; Kalinichenko and Pushchin, 2008). To the best of authors' knowledge, here we report the first description of the distribution of nNOS in the brain of a cetacean species.

Calbindin-D28k

The great majority of CB-ir neurons were small and medium sized polygonal and fusiform cells of layers II and IIIa (Fig. 6.3). Also few small neurons of the layer I were CB-ir. Occasionally,

lightly stained pyramidal neurons in layers IIIb-V and few ir-neurons in layer VI were observed. In two of three controls, a thick plexus of stained fibres, in correspondence of layers IIIb-V, was observable (Fig. 6.3 B). Also the fibres of the white matter showed strong immunostaining. In the neocortex of affected animals, the CB-immunoreactivity was visibly reduced. In layers II and IIIa there was a lower number of CB-ir cells, band of stained neuropil in layers IIIb-V disappeared and the immunoreactivity of the white matter was markedly reduced (Fig. 6.3 E-F).

In the cerebellum of control animals, almost all Purkinje cells showed and intense CB-D28k-ir localized in their soma and dendrites, which ran in the molecular layer, giving it a stained and dark appearance (Fig. 6.3 G-H). In the granular layer, few non stained somata, outlined by CB-D28k-ir axon terminals (Fig. 6.3 G), as well as some small weakly stained neurons (possibly UBCs and Lugaro cells), and intense immunoreactive fibres were observed. Additionally, many fibres of the granular layer showed CB-D28k-immunoreactivity. Differently from the cortex, in the cerebellum no eye-catching difference in the expression pattern between control and affected animals was noticed.

Calretinin

In the cortex of control striped dolphins, CR-immunoreactivity was mainly observed in strongly labelled polygonal and fusiform neurons of layers II and III (Fig.6.4 A-D). Occasionally, pyramidal neurons showed a weak staining. In most of the affected individuals (three of four) lower number of CR-ir cells were observed in layers II and III. In the cerebellum of controls, CR-ir cells were concentrated in the upper third of the granular layer (unipolar brush cells –UBCs-) (Fig. 6.4 E-F). Furthermore, the entire granular layer showed a weak and generalized labelling. In the cerebellum of individuals affected by meningoencephalitis there seemed to be a generalized reduction of CR-immunoreactivity with a mild decrease in number of ir-cells (Fig. 6.4 G-H).

nNOS

Neuronal NOS labelled cortical elements showed at least two grades of immunoreactivity, (strong and weak) (Fig. 6.5 B). In the cortex, nNOS-immunoreactivity was mainly localized in neurons of layers II-III and V-VI. The majority of darkly stained cells, multipolar, bipolar or round in shape, were concentrated in deeper cortical layers, mainly near the junction between grey and white matter and showed cytoplasmic immunoreactivity more evident at the periphery of the cell. On the other hand, lightly stained cells were much more numerous, had several shapes and sizes and were commonly found in layers II-IIIa and V. Pyramidal neurons seldom showed a weak labelling of the somata (Fig. 6.5 E). Occasionally, nNOS-ir varicosities and small interneurons were observed in lamina I. In the cerebellum, nNOS-immunoreactivity was not so intense as observed in some elements of the neocortex, and it was mainly localized in the molecular layer(Fig.6.5 F), in particular in more superficial cells (presumably stellate cells) and in neurons of its middle and deep portions (Fig.6.6 A-C). These latter cells could actually be basket cells, forming ir-arborizations around the initial portion of the axons of Purkinje cells (Fig. Fig. 6.6 B). Occasionally, in the granular layer, large nNOS-ir neurons, presumably Golgi cells (because of the size and morphology of the somata), were observed (Fig.6.6 D). Most Purkinje cells showed a certain degree of immunoreactivity, both in control and affected dolphins (Fig. 6.6 A). A difference in the intensity of nNOS-immunoreactivity between control and affected animals was more evident in the cortex (Fig. 6.5 C-D), while in the cerebellum there seemed to be no eye-catching variation (Fig. 6.6 E-F).

Quantitative analysis

The mean values of ir-area (%), obtained with ImageJ software, resulted almost always higher in controls than in animal affected by meningoencephalitis, but a statistically significant difference ($P<0.05$) existed only for the expression of CB-D28k in the cortex. Although no difference was noticed in light microscopy, ImageJ analysis pointed out higher percentages of CB-D28k-ir area in the cerebellum of affected animals compared with controls (Fig. 6.1).

Discussion

As far as we know, here we report the first description of nNOS-immunoreactivity distribution in the cortex and cerebellum of a cetacean species. As described in other species (Hashikawa et al., 1994; Gotti et al., 2005; Lee and Jeon, 2005), also in the striped dolphin, neurons show different degrees of nNOS-immunoreactivity, ranging from an intense dark staining to a weak light brown labelling. In the neocortex, most nNOS-ir neurons are non-pyramidal neurons, found almost in any layer with the exception of layer I where just occasionally some varicosities were observed. As previously reported in monkey (Hashikawa et al., 1994), mouse (Lee and Jeon, 2005) and rabbit (Lee and Jeon, 2005), the majority of darkly stained nitrergic neurons lie in layers V and VI while fainter cells belong to superficial layers (II and III). Comparison of the present and the literature data suggests that the localization of nNOS in the cetacean cerebellar cortex is similar to that in humans and rodents (Egberongbe et al., 1994; Rodrigo et al., 1994), and mainly localized in the stellate and basket cells of the molecular layer. Furthermore, we observed large immunoreactive somata in the granular layer, which could be Golgi cells. These cells are immunopositive for nNOS also in the sheep, rabbit, chick and goldfish (Bruning et al., 1995; Kalinichenko et al., 1997; Okhotin and Kalinichenko, 1999; Rodrigo et al., 2006), while negative in rodents (Chung et al., 2002; Rodrigo et al., 2004). An interesting finding was the labelling of most Purkinje cells in both controls and affected individuals. According to some authors, in the mouse (Cork et al., 1998; Gotti et al., 2005), and rat (Bredt et al., 1991; Vincent and Kimura, 1992; Blanco et al., 2010) cerebellum, Purkinje cells are nNOS-negative. Rodrigo and colleagues described few isolated nNOS-ir Purkinje cells in the vermis and parafloccular regions of the rat cerebellum (1994), and an occasional weak cytoplasmic staining of Purkinje cells in the cerebellum of sheep (2006). In some pathological conditions, Purkinje cells seem to become nNOS-ir (Saxon and Beitz, 1994; Rodrigo et al. 2004; Fernandez et al., 2007). For example, Fernandez and colleagues (2007) report that, in sheep affected by Scrapie, Purkinje cells, nNOS-negative in control animals, become immunoreactive in the initial

phase of the disease but the labelling disappears again in the terminal stage. In human cerebellum, the majority of Purkinje cells shows undeniable nNOS-immunoreactivity (Egberongbe et al., 1994). In the developmental rat cerebellum, Purkinje cells are known to express NADPH-diaphorase activity, which is the histochemical marker of nNOS (Yan et al., 1993). Hence, in most studied species, nNOS expression by Purkinje cells seems to be abolished during adulthood and able to reappear during tissue damage. Our results suggest that dolphin Purkinje cells, like human ones, seem to be able to maintain lifelong NOS producing capability.

Our results suggest a variation in the expression of some neuronal markers in the brain of striped dolphins affected by morbilliviral meningoencephalitis. Although no specific differences were observed between labelled cellular types in control and affected animals, a reduced number of immunoreactive elements, mainly in the cortex, was evident at a first evaluation in light microscopy and it was confirmed by ImageJ analysis. Nevertheless, statistical difference was detected only for the CB-28k-immunoreactivity in the cortex but it is known that small size samples makes it hard to find statistical significance. The decreased immunoreactivity could reflect a functional alteration in neuronal activity, a conformational change of the proteins (no longer recognized by the respective antibodies) or be the consequence of neuronal loss. Although we did not test any marker for neuronal damage and death, we would point out that in H&E sections, neuronal necrosis was not so massive to explain such a pronounced reduction in immunoreactivity as we observed. Thus, our hypothesis is that during the meningoencephalitis these proteins could be downregulated or could be affected by a conformational changes, Akaike and colleagues (1995) described a time-dependent decrease in nNOS activity and immunostaining in the brain of rats infected with Borna disease virus and rabies virus. They demonstrated that the activity of choline acetyltransferase (Chat) did not show any variation in infected brains, suggesting that the changes observed in nNOS activity and immunostaining were not ascribable to extensive neuronal death. Furthermore, just as we observed in the brains of dolphins with morbilliviral meningoencephalitis, they reported a more marked involvement of the cortex compared to the cerebellum.

Free calcium ions have several biological functions like stimulation of presynaptic neurotransmitter release (Neher and Sakaba, 2008) and gene transcription regulation (Lyons and West, 2011). An excessive and sustained increase in the intracellular levels of Ca^{2+} may activate different catabolic enzymes and consequently lead to necrosis or apoptosis (Yu et al., 2001; Orrenius et al., 2003). Evidence suggests that CBPs, especially those designated as buffers, may contribute to mitigate cellular damage during pathological conditions. The decrease in the CBPs immunoreactivity could reflect a modulation in the expression of the proteins induced by both host immune response and viral infection. This downregulation could initiate a vicious circle in which the decrease in the expression of fast calcium buffers, like CR and CB-D28k, could lead to neuronal damage.

A decrease in the expression of nNOS is reported in some CNS disorders, including those caused by viral infection (Akaike et al., 1995; Hooper et al., 1995; Fernandez et al., 2007). On the other hand, during the same pathological states, iNOS expression is usually enhanced and associated with high levels of NO. Although the role of NO in the nervous system seems to be quite paradoxical, constitutive NOS are generally assumed to be neuroprotective while iNOS activity is considered damaging. In fact, differently from nNOS and eNOS, iNOS produces high quantity of NO and in a Ca^{2+}-independent manner (Guix et al., 2005; Förstermann and Sessa, 2012). Further investigation is needed in order to clarify the role of the two isoforms during morbilliviral meningoencephalitis in Cetaceans. For instance, it would be interesting to verify if there is a correlation between variations in the expression of both nNOS and iNOS and the timing of the disease, comparing what happens in acute and chronic stages.

Limitations of the study

This study should be considered as a first preliminary overview on the variation of the immunoreactivity of some neural markers in the brain of striped dolphins affected by morbilliviral meningoencephalitis. Despite many efforts to make the study scientifically accurate, the research encompasses some technical/practical limitations. Firstly, as the sample size is small, it is difficult

to find significant relationships. In fact, statistical tests normally require a large sample size to ensure a representative distribution of the population. Furthermore, as frequently occurs with retrospective studies, the sampling of the different brain areas was not made following the same criteria over the 11-years period of collection. This implies, for example, that the samples of the cortex were not obtained from the same region for each animal. We tried to make our groups as homogeneous as possible, choosing the suitable animals also based on the conservation grade that they were assigned and selecting samples only from the freshest carcasses, with a conservation grade of 1 or 2. Nevertheless, among the animals chosen, a difference in the quality and preservation of the tissues could exist, and it could have affected the immunoreactive properties. Moreover, it is possible that also the form and timing of the disease (i.e. acute/subacute systemic, chronic systemic, chronic localized) influenced the expression of the proteins. Therefore, for the above-mentioned reasons, the results reported here, albeit original, have to be handled carefully and further investigation, with implementation of cases, is needed.

Tab. 6.1 Details of animals with morbilliviral meningoencephalitis

ID	Age	cons grade	Histological findings (CNS)	form
I-154/02	adult	1	**NS meningoencephalitis** (congestion, perivascular oedema, meningeal lymphocytic infiltrate, gliosis, perivascular cuffs)	chronic systemic
I-07/09	adult	2	**mild NS encephalitis** (meningeal and cortical haemorrhages and congestion; perivascular astrocyte ballooning; satellitosis and neuronophagy; choroid plexus hyalinization; neuronal lipofuscinosis; perivascular cuffs)	chronic localized to CNS
I-14/11	adult	2	**severe NS encephalitis**	chronic systemic
I-065/12	subadult	2	**NS mielomeningoencephalitis**	sub-acute systemic

The state of conservation of the carcasses was determined following the parameters and classifications established in the protocol published by the European Society of Cetaceans (Kuiken and García-Hartmann, 1991): level 1 (very fresh), level 2 (fresh), level 3 (moderate autolysis), level 4 (advanced autolysis) and level 5 (very advanced autolysis).

Tab. 6.2 Details of control animals

ID	Age	Cons grade	Cause of death
I380/01	young	1	By-catch
i087/02	adult	1	Aging –related pathologies
i232/10	adult	2	Trauma/ active stranding

The state of conservation of the carcasses was determined following the parameters and classifications established in the protocol published by the European Society of Cetaceans (Kuiken and García-Hartmann, 1991): level 1 (very fresh), level 2 (fresh), level 3 (moderate autolysis), level 4 (advanced autolysis) and level 5 (very advanced autolysis).

Tab.6.3 Details of antibodies used

Primary Antibodies	*Host*	*Code*	*Dilution*	*Source*
nNOS	rabbit	Ab 5380	1:300	Millipore
CR	mouse	6B3	1:1000	Swant
CB-D28k	rabbit	CB-38a	1:1000	Swant
Secondary Antibodies		*Dilution*		*Source*
biotinylated goat anti-rabbit (BA1000)		1:200		Vector
biotinylated goat anti-mouse (BA9200)		1:200		Vector

Abbreviations: nNOS neuronal nitric oxide synthase, CR calretinin, CB-D28k calbindin D-28k.

Figures

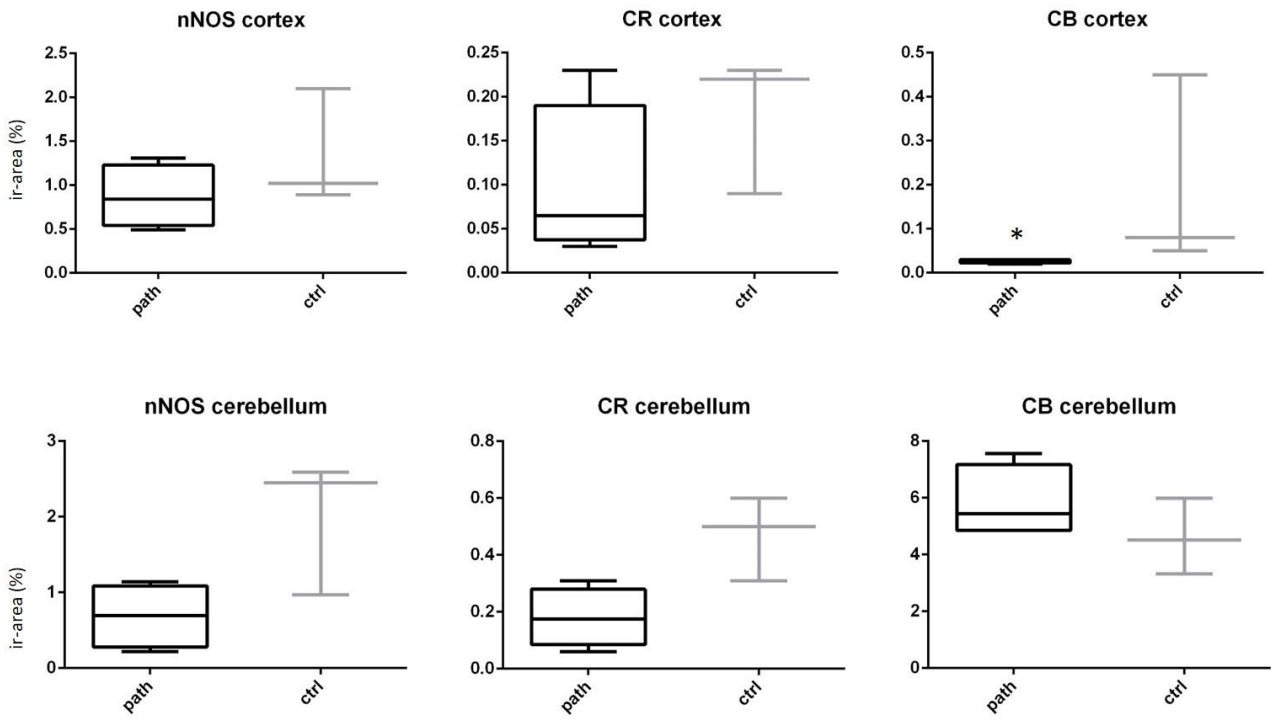

Fig. 6.1 Graphical representation of percentage of ir-area in controls (ctrl) and affected animals (path). Asterisk represent significant difference for $P < 0.05$.

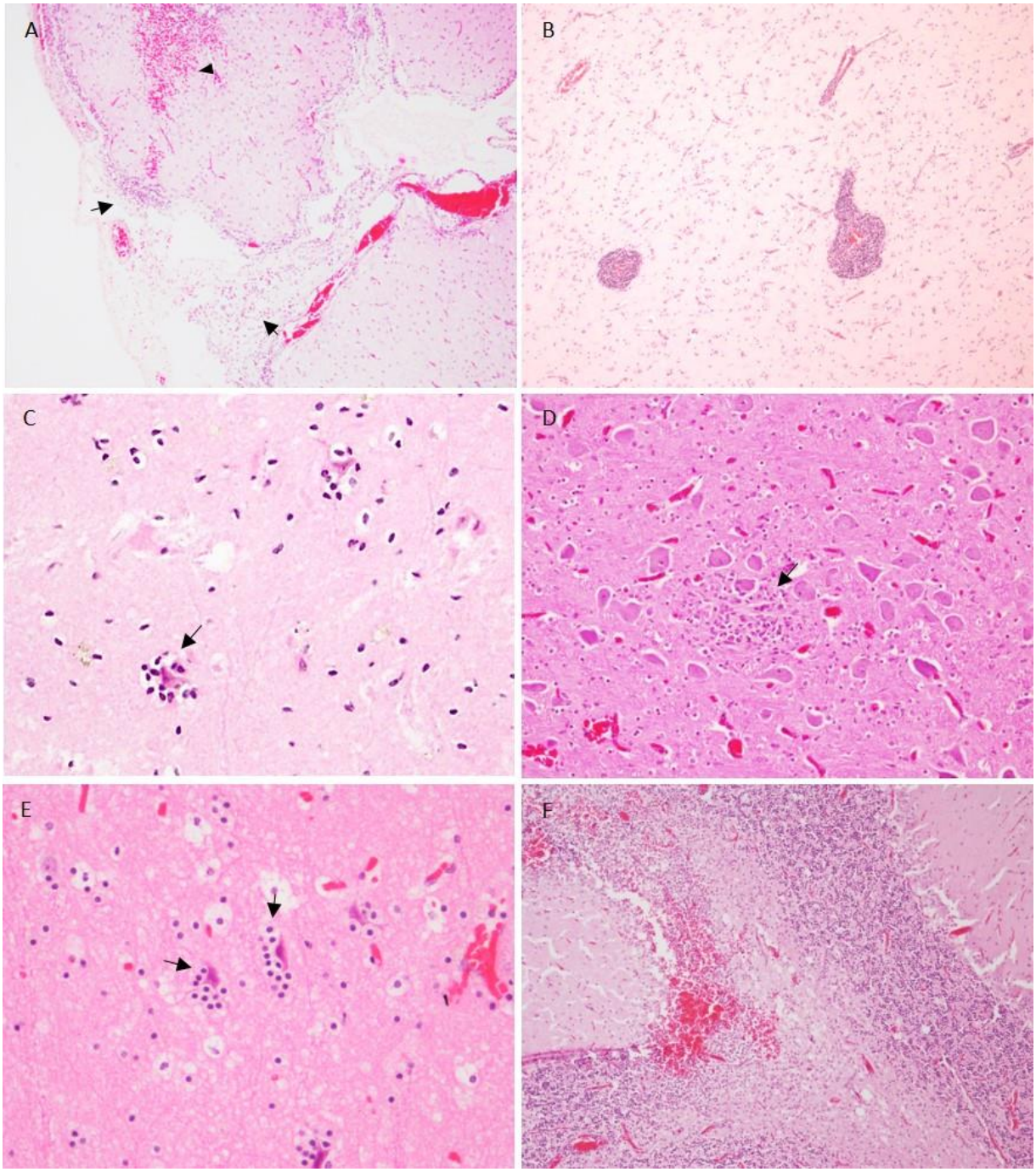

Fig 6.2 Photomicrographs showing typical histological finding in non suppurative (NS) meningoencephalitis in the brain of striped dolphins (*Stenella coeruleoalba*). A) lymphoplasmacytic meningitis in the cerebellum (arrows) and an area of hemorrhage (arrowhead) B) large perivascular lymphoplasmacytic cuffs in the cerebral cortex. C) black arrow shows satellitosis and neuronophagia in the cerebral cortex D) Pons, an area of focal gliosis (arrow). E) cerebral cortex, arrows show satellitosis surrounding «suffering» neurons. F) a focal area of hemorrhage in the cerebellum.

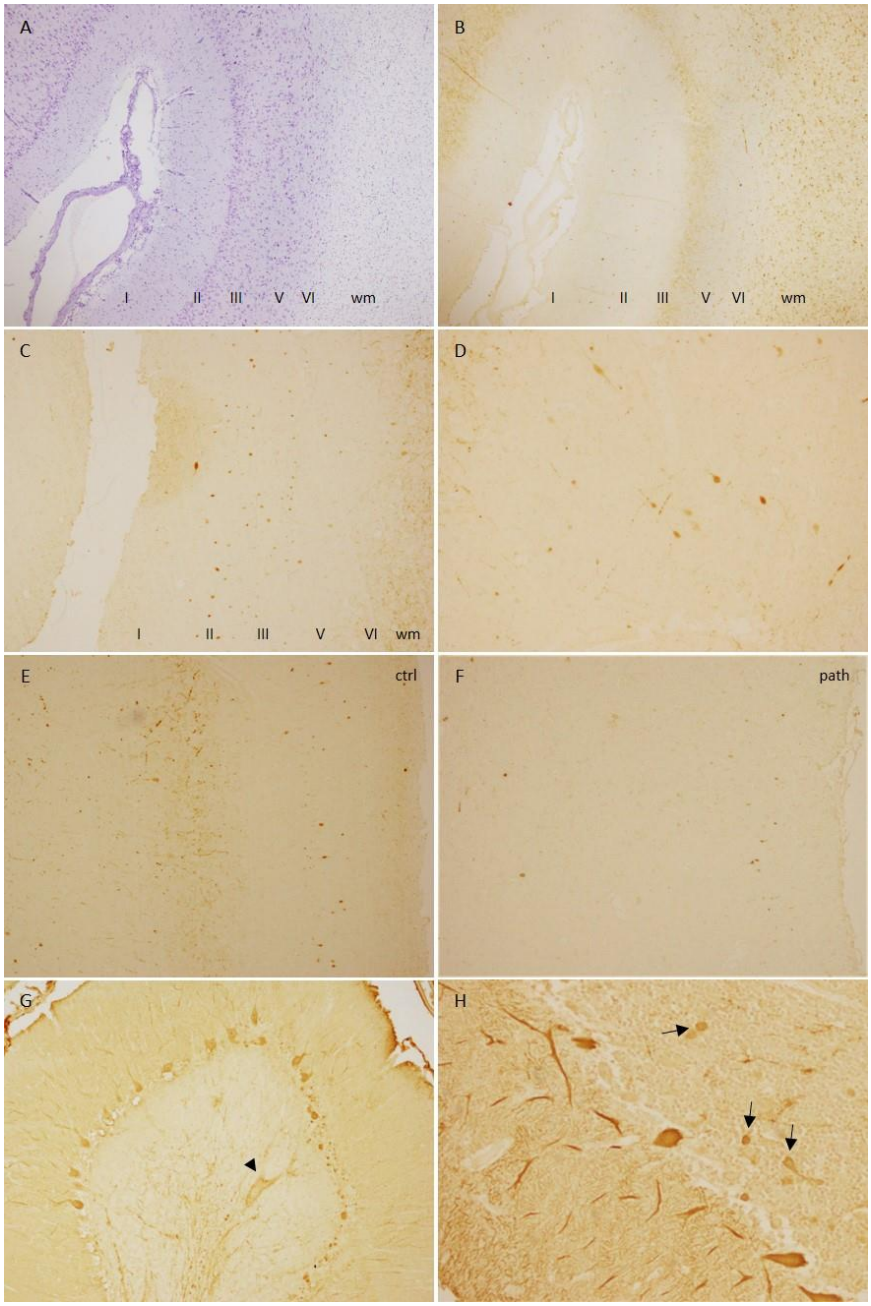

Fig 6.3 Photomicrographs showing calbindin-D28k (CB) immunoreactivity in the cortex and cerebellum of the striped dolphin (Stenella coeruleoalba). A-B) Overview of Nissl (A) and immunohistochemical (B) staining of the cortex. Note thick plexus of immunoreactive (ir-) fibres in correspondence of layers IIIb-V. C-D) The great majority of CB-ir cortical neurons are small and medium sized polygonal and fusiform cells of layer II and superficial layer III. E-F) Cortex. Comparison of the CB expression between controls (ctrl) (E) and affected (path) animals (F). Note the eye-catching reduction of the immunoreactivity in the cortex of striped dolphins affected by morbilliviral meningoencephalitis. G-H) CB immunoreactivity in the cerebellum. Note the intense staining of Purkinje cells and their dendrites running in the molecular layer. Arrowhead shows a non stained somata in the granular layer, outlined by CB-D28k-ir axon terminals. Occasionally some small weakly stained neurons of the granular layer (possibily unipolar brush cells –UBCs- and Lugaro cells) were observed (arrows).

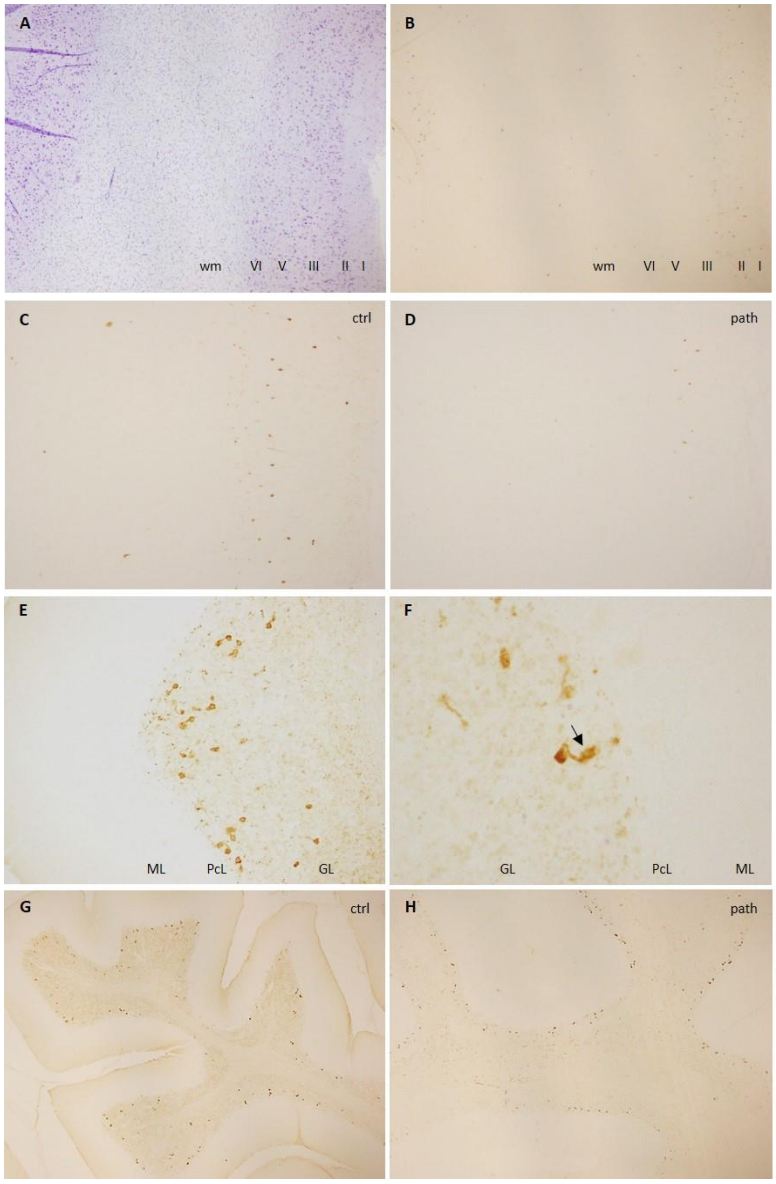

Fig.6.4 Photomicrographs showing calretinin (CR) immunoreactivity in the cortex and cerebellum of the striped dolphin (*Stenella coeruleoalba*). A-B) Overview of Nissl (A) and immunohistochemical (B) staining in the cortex. Note that immunoreactivity is mainly observed in strongly labelled small neurons of layers II and III, and occasionally I. C-F) Comparison of the CR expression between controls (ctrl) and affected (path) animals in the cortex (C-D) and cerebellum (E-F). Note the eye-catching reduction of the immunoreactivity in the brain of striped dolphins affected by morbilliviral meningoencephalitis. G-H) In the cerebellum CR-immunoreactivity is concentrated in the upper third of the granular layer. Ir-cells show the typical morphology of unipolar brush cells (UBCs). H) Arrow shows the brush-like termination of a UBC. These dendrioles represent the main synaptic apparatus of the UBCs and connect tightly with a single mossy fiber rosette in a glomerular-like structure (E-F). In the cerebellum of individuals affected by meningoencephalitis (path) there seem to be a generalized reduction of CR-immunoreactivity, when compared with controls (ctrl) (G-H). ML molecular layer, PcL Purkinje cells layer, GL granular layer

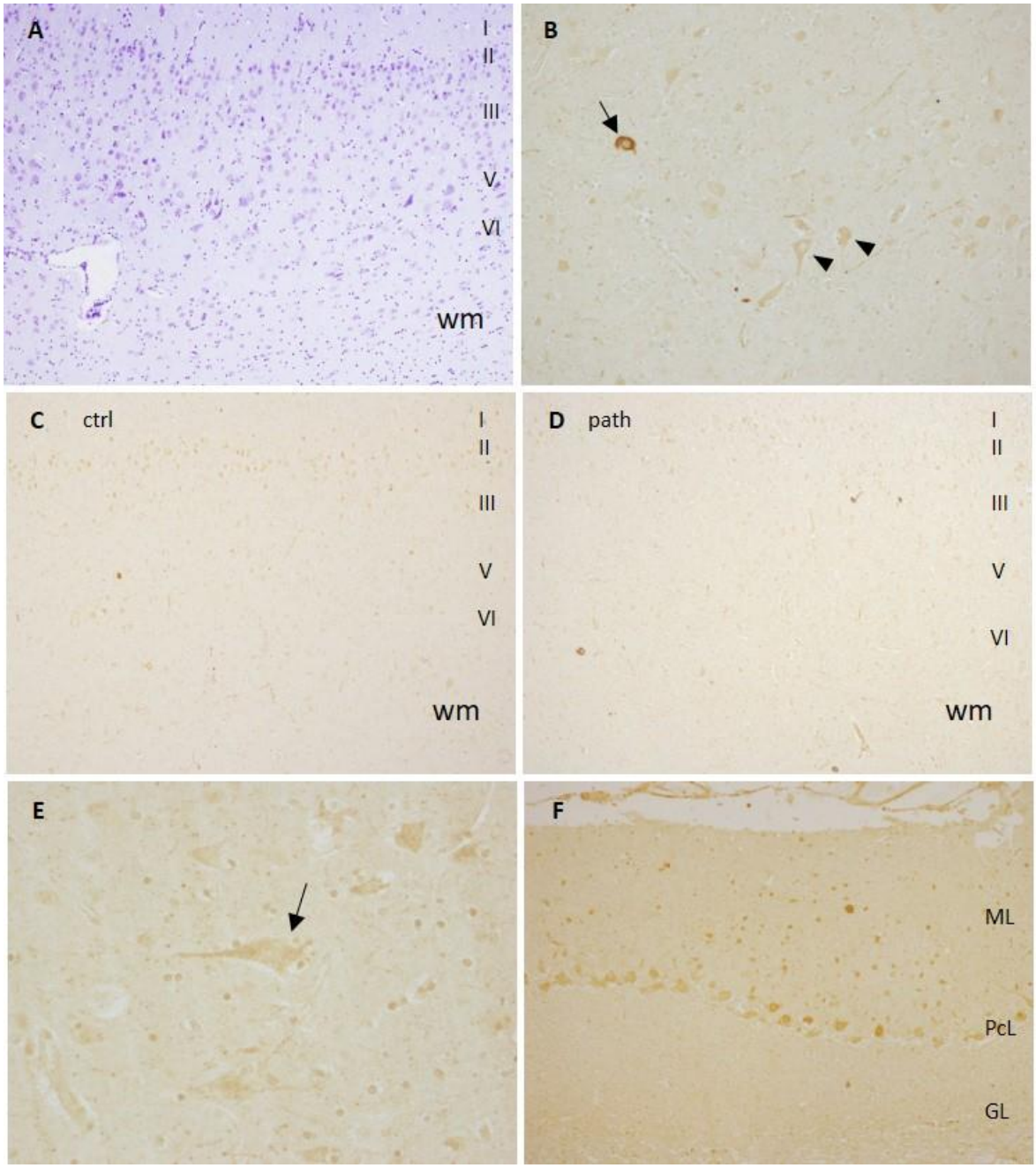

Fig.6.5 Photomicrographs showing neuronal nitric oxide synthase (nNOS) immunoreactivity in the cortex and cerebellum of the striped dolphin (*Stenella coeruleoalba)*. A-E) Nissl (A) and immunohistochemical (B-E) staining of the cortex. nNOS-immunoreactive (ir-) cells are mainly localized in layers II-III and V-VI. In all individuals, stained cells present at least two grades of immunoreactivity, strong (arrow) and weak (arrowheads) (B). Occasionally, pyramidal neurons show a faint labelling of the somata (E). There is a general reduction of nNOS immunoreactivity in the cortex of affected (path) dolphins when compared with controls (ctrl) (C-D). F) nNOS-immunoreactivity distribution in the cerebellum of the striped dolphin. Immunoreactivity is mainly localized in the molecular layer, in particular in more superficial cells and cells of its deeper portion.

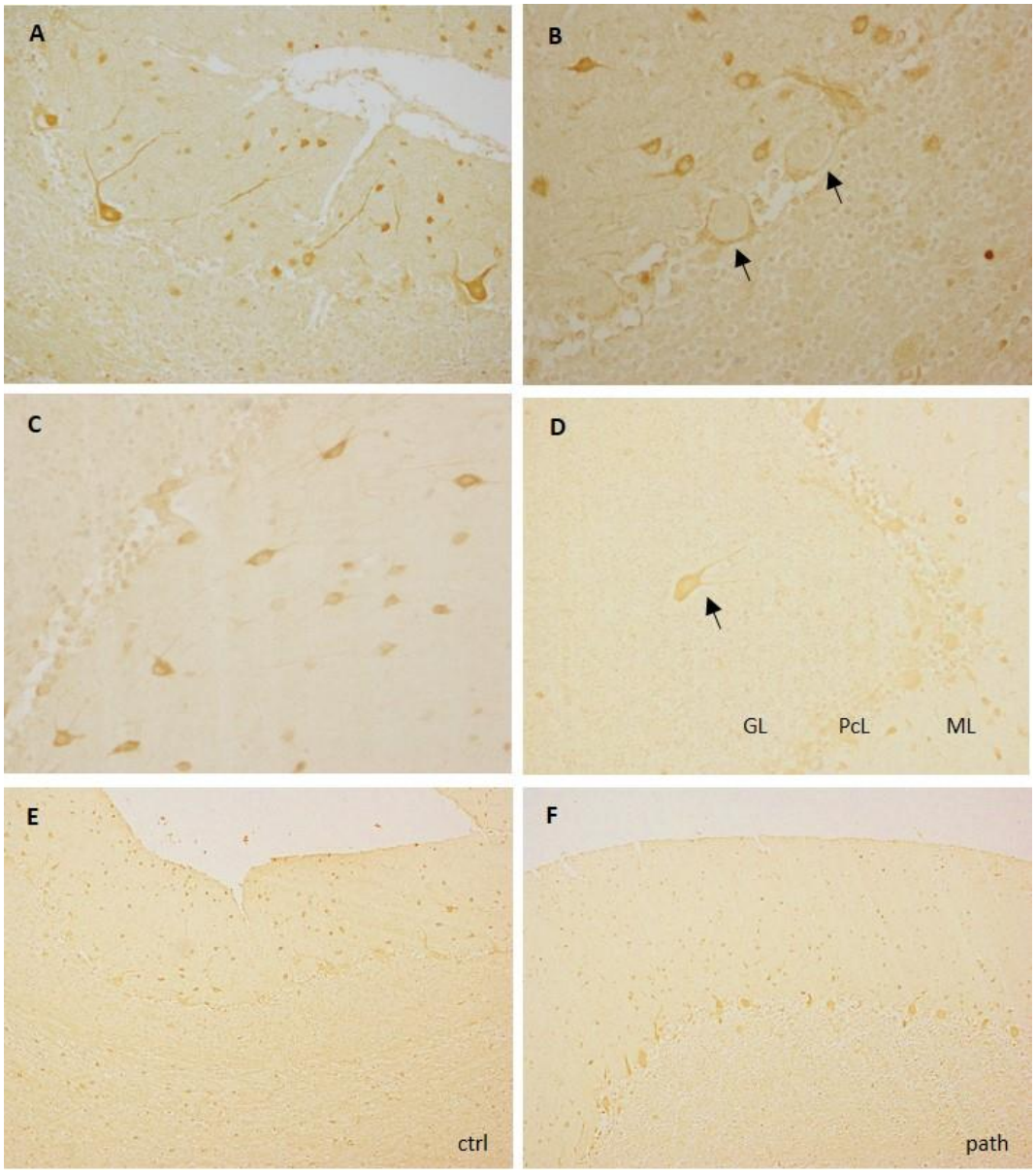

Fig.6.6 Photomicrographs showing neuronal nitric oxide synthase (nNOS) immunoreactivity in the cerebellum of the striped dolphin (Stenella coeruleoalba**). A-F**) nNOS-immunoreactivity distribution in the cerebellum of the striped dolphin. Immunoreactivity is mainly localized in the molecular layer, in particular in more superficial cells and cells of its deeper portion (presumably stellate ad basket cells, respectively) (C). Frequently, basket cells form ir-arborizations around the initial portion of the axons of Purkinje cells (B) (arrows). As described in the human cerebellum, most Purkinje cells show a certain degree of immunoreactivity (A). Occasionally, large nNOS-ir neurons, presumably Golgi cells, are observed in the granular layer (D) (arrow). Unlike the cortex, in the cerebellum no eye-catching reduction in the intensity of nNOS-immunoreactivity was observed between control (E) and affected (F) animals. ML molecular layer, PcL Purkinje cellslayer, GL granular layer.

CONCLUSIONS

Nowadays we are experimenting a global increasing need to conduct research as an important tool for conservation of animal populations and maintenance of their health status. In this context, marine mammals, in particular cetaceans, represent a demanding challenge. Their entirely aquatic lifestyle entails several impediments to investigations, widely related to their behavioural and ecological characteristics, which make difficult the collection of data and samples. Despite the crucial advances made in the last decades, many of the morpho-physiological peculiarities that characterize cetaceans have not yet been studied or fully understood. With this collection of studies, we tried to contribute to the current knowledge on dolphins neuroanatomy and neurochemistry. Two studies focused on the anatomical aspects of two different structures of the bottlenose dolphin CNS. In the first one we investigate the cytoarchitecture and the distribution of calretinin (CR) immunoreactivity in the lateral nucleus of the amygdaloid complex. We identified a higher number of subdivisions than those currently reported in rodents and (human and non-human) primates. Given this great heterogeneity, we hypothesized that dolphin lateral nucleus possess a high grade of complexity which might serve an outstanding capability of elaboration. Furthermore, we observed a strong presence of CR-ir fibres and neurons, mainly non-pyramidal inhibitory neurons. We postulate that CR-ir neurons could play an important role in the control of information flow in the amygdalar lateral nucleus, forming inhibitory synapses on calbindin-D28k interneurons, as reported for the human lateral nucleus. The second study was designed to investigate the expression of CGRP in the spinal cord and spinal ganglia of bottlenose dolphin. The distribution pattern that we observed results quiet similar to what described in other mammals. The large presence of CGRP in sensory and motor system of the bottlenose dolphin is suggestive of a heterogeneous functional picture for this peptide. Additionally, we pointed out that IB4 is not indicated as a marker of non-peptidergic neurons in the bottlenose dolphin. Concerning the peripheral nervous system, we carried on a study on nitrergic and substance P-ir neurons in the intestine of the bottlenose dolphin. Although the digestive system of Cetaceans present many peculiarities, still little is known on the enteric nervous system. With the present study, we provide the first report of enteric nitrergic

neurons in the intestine of a cetacean species and we also highlight some remarkable characteristics, as the total lack of colocalization between nNOS and SP in both mienteric and submucosal plexuses. As nNOS and SP are important mediators of intestinal functions and nitrergic population represents an important target of many neuroenteropathies, these data could be essential to understand possible functional differences and investigate motor intestinal dysfunctions/alteration. Lastly, we carried out a preliminary study focused on some neurochemical changes affecting the cortex and cerebellum of striped dolphins with morbilliviral meningoencephlitis. We explored the variations in the immunoreactivity of nNOS and calcium binding proteins (CABPs), quantifying and comparing the percentages of immunoreactive area in both controls and affected animals. The expression of nNOS and CABPs in the cortex resulted largely affected, with a statistically significative difference reported for the immunoreactivity of calbindin in the cortex. Despite several limitations, this study provides a first insight into some unreported neurochemical changes occurring during morbilliviral meningoencephalitis. We tried to interpret these variations in the light of what already reported about viral-mediated neuropathogenesis in other animals.

Concluding, to advance in the knowledge of cetacean species, new and original anatomical data represent an important piece of the jigsaw, which need to be implemented and completed by other disciplines. Although in the last years marine mammals have benefited from more public and scientific attention, we consider advisable that they were given more relevance in the veterinary medicine field, and that such attention would be characterized by an interdisciplinary approach.

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