A Comparative Study of Neuropeptide Y-Immunoreactivity in the Retina of Dolphin and Several Other Mammalian Species

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Shur-Tzu Chen, Ching-Liang Shen, Jiang-Ping Wang and Lien-Siang Chou (1999) A comparative study of neuropeptide Y-immunoreactivity in the retina of dolphin and several other mammalian species. *Zoological Studies* **38**(4): 416-422. The immunoreactivity of an antibody to neuropeptide Y (NPY) in the retina of striped (*Stenella coeruleoalba*) and Fraser (*Lagenodelphis hosei*) dolphins and several other species was studied and compared. In dolphin retina, moderate to intense immunostaining was observed primarily in the giant retinal ganglion cells and their dendrite-like processes, while ganglion cells with smaller somata were only weakly immunoreactive. In contrast, NPY immunoreactive cell bodies are mainly located in cells with small somata in the ganglion cell layer and cells resembling amacrine cells in the inner nuclear layer in rat retina. Positive immunostaining was also observed in the inner and outer plexiform layers of rat retina, a feature that was not found in dolphin retina. Interestingly, the overall pattern of NPY expression in the retina of dolphin is similar to that in dog but not in rat, or other mammalian species described previously. One possible explanation is that dolphin and dog are more active by day as compared with rat and cat which are considered to be nocturnal. The variable pattern of distribution of NPY observed in different species suggests that NPY plays a unique functional role, for instance, in the demand for higher levels of blood supply for visual connections through the influence of NPY, in the retinal system in a species-dependent manner probably related to animals' visual behaviors and environments.

Key words: NPY, Retina, Cetacean, Immunohistochemistry.

The retina of cetaceans is of great interest as it is potentially a model for studying retinal function and visually guided behavior of mammals that spend their entire lives in an aquatic environment. Previous studies have shown that dolphin retina is characterized by certain features which are not observed in most laboratory animals. For instance, it has been shown previously that dolphin retinas contain a small population of giant ganglion cells with unusually large somata and axons (Dral 1977 1983, Dawson et al. 1982, Li et al. 1983, Mass and Supin 1995). To our knowledge, giant ganglion cells similar to those in the dolphin have been reported only in elephant (Glickstein 1976), another large mammalian species with a living environment completely different from that of dolphins. In order to further understand and resolve the issue of whether dolphin retina is really different from those of commonly used laboratory animals, we sought to characterize the biochemical properties of cells in dolphin retina and compare them with those of other species including rodents and carnivores. A regulatory neuropeptide identified in the retina, neuropeptide Y (NPY), was used as a marker in this study, because it appears to play an important role in neuronal function, and because the NPY system in the retina has been well documented in a variety of species.

NPY is a 36-amino acid amidated polypeptide that was first isolated from porcine brain and characterized by Tatamoto and his collaborators (Tatemoto et al. 1982). The high levels of NPY-immunoreactive nerves observed in the vascular system and those

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coexisting with catecholamines in sympathetic nerves indicate that NPY can influence cardiovascular function by mean of its effect on autonomic transmission (Wharton and Gulbenkian 1989). Previous studies have also suggested that the wide distribution and specific expression in both central and peripheral nervous systems point toward the molecule being a neurotransmitter candidate, or a regulator of neurotransmission (Allen et al. 1983, Lundberg et al. 1984, Shen 1987, Hung and Shen 1994, reviewed by Wahlestedt and Heilig 1994). In the retina, NPY immunoreactivity has been found in amacrine cells and the inner plexiform layer of several species such as fish, frog, chicken, cat, pig, baboon, and human (Bruun et al. 1986 1991, Torqvist and Ehinger 1988, Hutsler et al. 1993, Hutsler and Chalupa 1994). Information about NPY distribution in rat or rodent retina, however, remains controversial as conflicting results have been reported, with some studies reporting a lack of NPY in the retina, while others found NPY-immunoreactive amacrine and ganglion cells (Allen et al. 1983, Lundberg et al. 1984, Shen 1987, Hung and Shen 1994, reviewed by Wahlestedt and Heilig 1994). In the retina, NPY immunoreactivity has been found in amacrine cells and fish, frog, chicken, cat, pig, baboon, and human (Bruun et al. 1986 1991, Torqvist and Ehinger 1988, Hutsler et al. 1993, Hutsler and Chalupa 1994). Information about NPY distribution in rat or rodent retina, however, remains controversial as conflicting results have been reported, with some studies reporting a lack of NPY in the retina, while others found NPY-immunoreactive amacrine and ganglion cells.

Considering that the rat is a commonly used laboratory animal for studying neuronal organization and function, and an in vivo model for studying retinal development and pathology in our laboratory (Chen et al. 1994a,b 1996 1998), we are particularly interested to compare the activities of the NPY system between rat and dolphin, 2 species with completely different visual motor behaviors and environments. In addition to differences of living on land or in the sea, dog retina was used to compare differences of visual behaviors with the rat and cat which are nocturnal animals, while dolphin and dog are probably not. In this study we describe the occurrence and morphology of NPY-immunoreactive neurons in the retinas of 2 species of cetaceans and identify a specific population of neurons which strongly immunoreact with this molecule. We also compare patterns of NPY expression between marine and terrestrial mammals, and show that the distribution of neuronal substances varies considerably between different species.

### MATERIALS AND METHODS

#### Experimental animals

Two *Stenella coeruleoalba* (striped dolphin) and 1 *Lagenodelphis hosei* (Fraser dolphin), all mature females, 200 to 240 cm total length (mouth to fluke notch), were stranded and died from wounds in March and April 1998 at beaches near Taichung and Pingtung Harbors in the west and south of Taiwan, respectively. The eyes were removed within 1 to 4 h of death and the retinal layer was separated from the choroid layer and thick sclera, and then immersion-fixed (fixative: 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4) for 6 h. Two other mammals were used, including rodents (2 adult Sprague-Dawley rats) and carnivores (2 young dogs) killed with an overdose of sodium pentobarbital (> 60 mg/kg, i.p.). While the young dogs were selected from animals whose cause of death was not related to disease of the visual or nervous system, the rats were raised by the Animal Resource at National Cheng Kung Univ. Medical College. After intracardial perfusion with the same fixative as above, the eyes of the rats and dogs were dissected. A portion of the retinas of different species was prepared as wholemounts by dissecting them free in phosphate buffer saline (PBS), carefully cleansing them of vitreous humors, and flattening them under coverslips in a fixture for 24 h. This had the effect of permanently flattening the retina, and improving the evenness of subsequent staining and labeling procedures. Finally, frozen sections were prepared either from retinas prepared initially as wholemounts or from whole eyeballs (Chen et al. 1994b).

#### Immunohistochemistry

Frozen sections cut at a thickness of 20 µm were collected on gelatin-coated slides, and then processed for immunohistochemistry for NPY (Incstar, RAS-7172N, diluted to 1: 2000; Sigma, St. Louis, MD, USA, N9528, diluted to 1:1000). While 1 antibody was raised against a human NPY peptide and peptide YY (PYY, human), another was raised against synthetic porcine NPY with a marked sequence homology with PYY and pancreatic polypeptide. Both polyclonal antibodies have been used to detect NPY in a wide range of species, and no cross-reactivity in dot blot with substance P, vasoactive intestinal peptide, somatostatin, or calcitonin gene related peptide was found. Immunostaining for some sections as controls was abolished by either: (i) omitting the primary antiserum or (ii) incubating the sections in antiserum that had been preabsorbed with 1 µM synthetic peptide antigen NPY (Shen 1987, Hung and Shen 1994). Retinal sections were then reacted by using the avidin-biotin-complex procedure and developed by the glucose oxidase nickel-diaminobenzidine enhancement method (Chen et al. 1994a,b 1996 1998). No positive immunoreactivity was observed in the antigen-preabsorbed control
sections. Some adjacent sections were stained with cresyl violet to reveal the cytoarchitecture of the retinal tissue. Next, flattened wholemounts were pre-treated with 0.3% Triton in PBS for 15 min, and then processed through the same steps as sections, with the incubation times doubled.

RESULTS

Histological features in striped and Fraser dolphins revealed that cresyl violet-stained retina had distinct sublayers as observed in other vertebrates, including 3 cellular subpopulations, the ganglion cell layer (GCL) and inner and outer nuclear layers (INL, ONL), and 2 synapse-rich zones, the inner and outer plexiform layers (IPL and OPL) (Fig. 1A). Also observed herein were the giant neural cell systems of large ganglion cells with somata dimensions ranging up to 70 µm in diameter in the GCL, as described in retinas of other cetaceans (Fig. 1A, B). These giant cells had various shapes and sizes (30-70 µm). Round, oval, and polygonal neurons, indicated by the presence of several processes, were observed by Nissl staining. In general, these cells stained faintly to moderately as compared with small dark cells (neurons or glia) distributed in the GCL, INL, and ONL. The large blood vessels were mainly located in the innermost part of the retina (Fig. 1B, C), while small vessels were found within the retinal sublayers.

The NPY immunoreactive cells in dolphin retina appeared to be located primarily in the GCL, including medium to large cells (20-70 µm) (Fig. 1C-F). These neurons were generally polygonal or oval in shape. Fasciculus of optic axons with a thick diameter stained moderately as shown in both retinal whole-mounts and sections (Fig. 1D). In some regions, the processes of NPY-positive ganglion cells extended to the IPL, whereas only a few cells in the INL were immunoreactive (Fig. 1F). Additionally, only faint immunoreactivity was observed in the IPL, OPL, and ONL in dolphin retina (Fig. 1C, D, F). In general, the overall patterns of NPY immunoreactivity resembled each other in the 2 dolphin species (striped and Fraser dolphins).

In dogs, most cells in the GCL were medium- to large-sized (15-40 µm), and nearly all of these cells were NPY positive (Fig. 1G, H). Although giant ganglion cells (> 40 µm) were absent from dog retina, we observed that some NPY-positive neurons had large somata and extended their processes to the IPL (Fig. 1H). Under high magnification, the pattern of NPY expression in the GCL in dog was similar to that of dolphin retina (Fig. 1F, H). In the IPL of dog retina, the immunoreactivity was weak, like that in the dolphin while moderate immunostaining was observed in the IPL of rat retina (Fig. 1I). Furthermore, the NPY-positive cells distributed in the inner INL bordering the IPL, which resembled amacrine cells, were identified in rat retina, but not in that of dog or dolphin. In some regions of rat retina, NPY-immunoreactive cells were found in the outer part of the INL, resembling horizontal cells, according to their location and immunoreacted processes in the OPL. In the GCL of rat retina, the staining intensity of immunoreactivity was moderate to intense (Fig. 1I). In addition, in retinal tissues of various mammals (dolphin, dog, rat) immunostained with a different NPY antibody (Sigma, St. Louis, MD, USA, N9528), however, no difference was observed as compared with the expression of NPY immunoreactivity described above (reaction with antibody from Incstar, RAS7172N).

DISCUSSION

The most significant findings of our investigation on the distribution of NPY immunoreactivity in dolphin retina are summarized below. First, the study confirms that NPY-immunoreactive perikarya and nerve fibers are present in the retina of cetaceans as reported in other mammals and non-mammalian vertebrates. Second, we initially demonstrated that NPY immunoreactivity in dolphin retina is mainly expressed in large ganglion cells, which are part of the giant neural cell system. The pattern is not the same as that described previously in several terrestrial mammals or other non-mammalian species. In contrast, we found that the distribution and localization of NPY-containing cells in dolphin resembles that in dog retina, and this may reflect similarities in their visual behaviors.

Previous studies of the distribution of NPY in retina of fish, frog, and cat as well as in primates revealed that NPY-staining cells are primarily located in the amacrine cells of the INL, while some small ganglion cells also immunoreacted in some species, such as human (Osborne et al. 1985, Bruun et al. 1986 1991, Tornqvist and Ehinger 1988, Hutsler and Chalupa 1994). The early appearance of NPY in the amacrine cells of cat retina suggests that this molecule participates in establishing ganglion cell mosaics and in transferring visual information (Hutsler and Chalupa 1994). The NPY immunoreactivity strongly present in the different sublaminae of the IPL in retina of cat and non-mammals (i.e., toad and lizard) suggests that NPY-containing amacrine cells are in-
Fig. 1. Photomicrographs showing the cytoarchitecture and immunohistochemistry of NPY-immunoreactivity in retina of dolphin (A-F), dog (G, H) and rat (I). (A) Fraser dolphin retinal section stained by cresyl violet shows well-laminated structures. The medium and large ganglion cells in the GCL stained weakly to moderately, while small cells in the INL and ONL are considerably darker. (B) Numerous large ganglion cells are observed after focusing on the GCL in this wholemount retina obtained from a Fraser dolphin. The blood vessels are usually located on the inner surface of the retinal layer (arrowhead, same in C, red color originally). (C) NPY immunoreactivity of the GCL of striped dolphin retina is principally distributed in medium and large cells. (D) Immunoreactive nerve fibers were also detected on the optic nerve fiber layer (arrows). (E) NPY-positive neurons are shown in a retinal wholemount of Fraser dolphin. At higher magnification, immunoreactive ganglion cells extend their processes to the IPL (arrowhead, F). In some retinal regions, NPY immunoreactivity appears in the INL. (G) In dog retina, NPY immunoreactivity is mainly expressed in the GCL. At higher magnification (H), the pattern of NPY-positive ganglion cells resembles that in dolphin retina. (I) In rat retina, NPY immunoreactivity can be found not only in the GCL but also in the IPL and ONL. Note that the immunoreactive cells in the GCL (arrow) are small. The small NPY-positive cells in the INL are largely located on the inner part, resembling amacrine cells (arrowhead). Scale bars = 100 µm.
volved in different synaptic circuits (Hutslcr and Chalupa 1994, Straznicky and Histcock 1994, Zhu and Gibbins 1995). On the other hand, dopaminergic and cholinergic neurons occur among the amacrines of most species (Ehinger 1983, Stone et al. 1989), and NPY has been reported to co-express with these neurons in the central visual system (Parnavelas et al. 1989). These studies demonstrate that NPY and tyrosine hydroxylase, as well as choline acetyltransferase immunoreactivities are principally present in the INL, sending processes to the IPL associated with the on-off organization. The present observations in dolphins, however, identify only a few or none of the immunoreactive cells in the INL, and processes extending from NPY-positive cells are diffused in the IPL in dolphin retina. These results suggest that perhaps NPY has other functions than the regulation of the retinal circuit.

The finding that NPY immunoreactivity appears in the GCL and not in the inner part of the INL in dolphin is suggested to be due to fixation (immersion/perfusion) of the retinal tissue. Most tissues of experimental animals in previous studies were prepared via a perfusion process and were not fixed post-mortem as in the present dolphin study. However, the NPY distribution in dog retina, dissected following perfusing fixation treatment, resembled that in the dolphin, indicating that NPY immunoreactivity was not influenced by the preparation of retinal tissues. Instead, the various expressions might be due to molecular differences in different species, since previous studies have indicated that human NPY differs somewhat from porcine NPY (Minth et al. 1984). As NPY expression reacting with 2 different antibodies has shown a very similar pattern in this study, whether NPY immunoreactivities extracted from cetacean and certain mammals have different molecular forms requires further study.

In cat retina, NPY appears in the gamma-type small retinal ganglion cells that project to the superior colliculus and C layers of the lateral geniculate nucleus (Hutslcr et al. 1993). Since 2 carnivores, i.e., cat and dog, are generally considered to have similar populations of ganglion cells, the observation obtained from dog retina has shown that the neurochemical expression is varied, because NPY is primarily distributed in the medium and large ganglion cells. It is possible that this is related to the fact that cat is a nocturnal animal like rat, while dog and dolphin are not. In view of this difference, it might be important to examine the retinal projection of ganglion cell subtypes in the cetacean’s central pathway to clarify the chemical function of the visual circuitry. For example, a very recent study has shown that the topographic distribution of calcium-binding protein varies in the visual system between dolphin and monkey (Glezer et al. 1998). Moreover, because NPY is well known to influence cardiovascular function via an autonomic effect (Wharton and Gulbenkian 1989), the blood supply of the retinal and visual system in aquatic mammals requires further investigation for elucidating the neurochemical features of the various systems and species.

Finally, since in most mammals the upper limit of behavioral visual acuity is imposed by the size and density of ganglion cells, conduction velocity and visual acuity are considered to be higher in animals containing large retinal cells. As mentioned above, although the large ganglion cells in elephant retina were proposed to be the result of the need to connect long retinal targets (Glickstein 1976), the functional significance of large ganglion cells in aquatic mammals remains obscure. In conclusion, most large ganglion cells in dolphin retina displayed NPY immunoreactivity, while the INL or amacrine cells revealed low levels of NPY, different from that previously described in terrestrial mammals and non-mammalian vertebrates. Despite the rich distribution of NPY immunoreactivity in the retina, and varied physiological functions of this peptide in the central and peripheral nervous systems, little physiological work combining neuroanatomic investigations has been performed in the eye.

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REFERENCES


神經勝肽 Y 在海豚及陸上哺乳類之視網膜之免疫反應研究

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本實驗探討及比較神經勝肽 Y(NPY)在鯨豚類及數種陸上哺乳類視網膜的表現情形。結果顯示，具有 NPY 免疫化學反應性之細胞，在海豚視網膜中主要分布在大型網膜神經節細胞，此種巨細胞(30~70 µm)在齧齒、食肉與靈長類是不存在的。前人發表之人類及實驗動物(例如大白鼠與猫科動物)，含 NPY 的細胞主要分布在內核細胞群，但是犬科，我們發現 NPY 細胞主要為中大型神經節細胞(20~40 µm)，而且表現型式較似鯨豚類。此研究證明，神經勝肽的分布因動物種類而異，而 NPY 在海豚視網膜的特殊表現意味著，含 NPY 之巨大型細胞在其生活環境有其特別的視覺傳導作用。

關鍵詞：神經勝肽 Y(NPY)，視網膜，鯨豚類。

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