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Dopamine beta hydroxylase and p73 expression in the Compound Eyes of the *Branchipus schaefferi* and *Artemia parthenogenetica*

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Summary
Dopamine beta hydroxylase and p73 expression in the Compound Eyes of the *Branchipus schaefferi* and *Artemia parthenogenetica.*
The anostraca *Branchipus schaefferi* belongs to the most endangered crustacean species in Europe. The *Branchipus schaefferi* inhabits small, temporary ponds with turbid water; the periods of desiccation are inundated with quiescent eggs. The *Artemia parthenogenetica* lives in coastal salt water or hypersaline water.
Anostraca are equipped with an unpaired, median light sensitive organ, a nauplius eye and a pair of facetted lateral eyes (compound eyes). Dopamine beta hydroxylase (DBH) has been described in different ocular tissues. Protein p73 is a complex protein with a variety of isoforms and an equilibrium between p73 isoforms is necessary for the survival and maintenance of sympathetic neurons. Anti-p73 and anti-DBH were used at the level of the compound eyes and optic ganglia of two anostraca and immunohistochemistry and western blot were used. DBH was expressed in retinal cells of the eyes of both anostraca, and p73 was present in retinal cells of the *Artemia* but at the crystalline and corneal level of the *Branchipus* compound eyes. This dissimilar localisation of p73 could mean a different role of p73 in the eyes of the two anostraca specimens.
Key words: anostraca, *Branchipus schaefferi*, *Artemia parthenogenetica*, Dopamine beta hydroxylase, p73, compound eyes.

Resumen
Expresión de dopamina beta hidroxilasa y la p73 en los ojos compuestos de la *Branchipus Schaefferi* y *Artemia parthenogenetica*
El Anostraca *Branchipus Schaefferi* pertenece a las especies de crustáceos más amenazadas de Europa. El *Branchipus Schaefferi* habita pequeños charcos, temporales con agua turbia; los períodos de desecación son inundados con huevos quiescentes. *La Artemia parthenogenetica* vive en agua salada o agua hipersalina en la costa.
Estos Anostracas están equipados con un órgano impar localizado en el medio, un ojo naupliar y un par de ojos laterales facetadas (ojos compuestos). La dopamina beta hidroxilasa (DBH) se ha descrito en diferentes tejidos oculares. La p73 es una proteína compleja con una variedad de isoformas y un equilibrio entre las isoformas de p73 es necesaria para la supervivencia y el mantenimiento de las neuronas simpáticas. Anti-p73 y anti-DBH se utilizaron técnicas inmunohistoquímicas y técnicas de Western blot a nivel de los ojos compuestos y ganglios ópticos de los dos Anostraca. DBH se expresó en células de la retina de los ojos de ambos Anostraca, y p73 estaba presente en células de la retina de la *Artemia*, pero en el cristalino y en la córnea de los ojos compuestos del *Branchipus*. Esta diferente localización de la p73 podría significar un papel diferente de p73 en los ojos de los dos especímenes de Anostraca.
Palabras clave: anostraca, *Branchipus schaefferi*, *Artemia parthenogenetica*, dopamina beta hidroxilasa, p73, ojos compuestos

Introduction

The anostraca *Branchipus schaefferi* belongs to the most endangered crustacean species in Europe. The *B. schaefferi* inhabits small, temporary ponds with turbid water. The *Artemia parthenogenetica* lives in salt water or hypersaline water near the coast of the islands of Fuerteventura and Lanzarote (Canary Islands) [6,7, 19, 26]. The anostraca nervous system (NS) consists of a dorsal brain, paired circumenteric connectives, and double, ventral nerve chords with segmental ganglia. The brain is a mass of translucent tissue surrounding the naupliar eye in the anterodorsal part of the head. The localization of neurotransmitters and neurohormones in the

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central nervous system has been studied by histochemical and by immunohistochemical techniques [2, 11, 14,15, 30]. Furthermore, the structure and development of the adult anostraca compound eyes and optic neuropils have been studied by several authors [9, 10, 12, 18, 20]. Thus, the anostraca sensory system includes the median naupliar eye and two stalked, lateral compound eyes [1,8, 23]. On the other hand, catecholamine has recently been identified in adult invertebrate animals, including arthropods, molluscs, and nematodes [5, 13, 25, 31]. Dopamine beta hydroxylase (DBH) has been described in different ocular tissues [4, 24]. The p73 protein belongs to the tumor suppressor protein p53 family with which it shares a strong structural similarity. p73 is a complex protein with a variety of isoforms. The transactivating isoforms (TA) are able to transactivate the p53 gene target and induce apoptosis, whereas the N-terminally truncated isoforms (ΔN) have anti-apoptotic activities, and an equilibrium between both isoforms is necessary for the survival and maintenance of sympathetic neurons [21, 22, 27, 29]. The aim of the present work is to compare the compound eyes and optic ganglia of two anostraca species: fresh water *B. schaefferi* and hypersaline water *A. partheno-genetica*, using antibodies against peptides and proteins involved in the development of sympathetic ganglion cells.

Material and methods

Experimental animals

Ten specimens of *B. schaefferi* collected in Puerto del Rosario (Fuerteventura), from the temporary rockpools that were formed by rainwater in the months of December 2005 and January 2006 and ten specimens of *A. parthenogenetica* were collected from the coastal waters around Fuerteventura. Four specimens from each group were fixed by immersion in Bouin's fluid, dehydrated and embedded in paraffin under standard conditions. Specimens were cut into four serial sagittal sections of a thickness of 7 µm. One of the serial coronal sections was stained by the hematoxylin-eosin method (H-E) (Fig. 1).

Immunohistochemistry

The polyclonal antibodies against TAp73 (Ab 14430, Abcam, Cambridge, UK) and DBH (AB 1585 CHEMICON International Inc.) were used as the primary antibodies. The sections at the level of the compound eyes and optic ganglia of two anostraca specimens in each group were simultaneously incubated in the same coupling jar and each jar contained: anti-TAp73 1:500 and anti-dopamine beta hydroxylase (DBH) 1:1000. Incubation was for 24 h at room temperature, followed by "DAKO" StreptABCcomplex/HRP Duet, Mouse/Rabbit procedure. The peroxidase reaction product was visualized using diaminobenzidine reaction. The primary antibodies were omitted to validate the control method specificity (Fig.4). The immunohistochemistry slides were converted to digital images by using a LEICA DMRB photomicroscope with an LEICA DC 300 F camera (Germany). Image analysis was completed in Image J (v. 1.43 u, NIH, Bethesda, MD, USA).

Western blot

After quantification of the protein concentration in the tissue extracts of specimens from six *B. schaefferi* and six *A. parthenogenetica.* 10 μg of total proteins was added to the sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol plus 0.2% bromophenol blue), denatured at 95 °C for 5 min and after rapid centrifugation was fractionated by SDS–PAGE using 10% polyacrylamide gel (Bio-Rad, Hercules, CA, USA) according to Laemmli [16]. The proteins were then transferred for 2 h to transfer buffer (25 mM Tris-Base, 192 mM Glycin and 20% methanol) at 4 °C at 200 mA constant on a PVDF filter with pores of 0.45 µm in diameter (Bio-Rad, Hercules, CA, USA). Western blotting experiments were performed using anti-p73 1:1000 and anti-DBH 1:2000 overnight at 4ºC as a primary antibodies. Anti-mouse IgG labelled with peroxidase (PIERCE) was used as the secondary antibody at a dilution of 1:8000 for 1h 45min at room temperature. The peroxidase reaction products from western blot were visualized by chemiluminiscence (Thermo Scientific™ Pierce antibody collection, SuperSignal™ chemilumi-nescent substrates, BCA). The primary antibodies were omitted to validate the control method specificity. Band intensities were quantified using the software ImageJ.

Statistical analysis

Intensities from images were semi-quantitatively analyzed by densitometry (ImageJ software, NIH Image). The "Mean Gray Value" was measured from all stained tissue and membranes. This value gives the average stain intensity in grayscale units for all threshold pixels.

A one-way ANOVA was used for data comparison between groups, which was conducted using the IBM SPSS statistic 19 software where data were considered as statistically significant at p-values < 0.05.

Results

H-E staining

The structure of the compound eyes (ce) in both of the two anostraca specimens were similar (Fig.1), where corneas (co), crystalline cones (cr), distal, proximal and reflectance retinal cells (rc) and nerve fibers to the optic (nfo) nerve can be distinguished.

Immunohistochemistry

DBH-immunoreactivity (DBH-ir) was expressed in the lateral compound eyes of the *B. schaefferi* and

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A. parthenogenetica, where the DBH-ir was mainly located in the distal, proximal and reflectance retinal cells (rc) and nerve fibers to the optic (nfo) nerve. The DBH-ir expression in the compound eye was fairly similar in both specimens (Fig.2,5). However, the DBH-ir was high in the distal retinal cells (Fig.2B,5) in the *Artemia* compound eye. On the other hand, p73-immunoreactivity (p73-ir) was found in both anostraca species (Fig.3,5) but was located in different places. p73-ir was found around the crystalline and cornea and on the border between retinal cells and optic nerve fibers in the *B. schaefferi* (Fig.3C,D,), whereas p73-ir was mainly found in retinal cells in the *A. parthenogenetica* (Fig.3A,B).

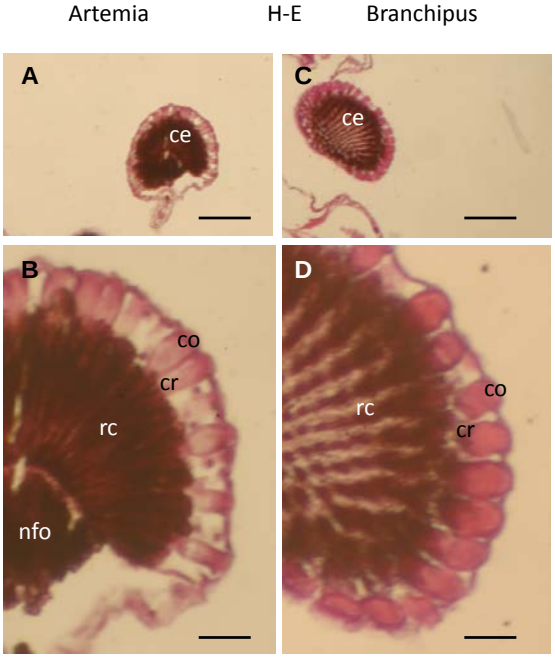

Figure 1.
Sagittal section of the compound eye (ce) of *A. parthenogenetica* and *B. schaefferi* stained with hematoxylin-eosin (H-E). A, B, ArP; C,D BrS. Scale bars: 200 µm in A, C; 20 µm in B,C.
ce=compound eye, co=cornea, rc= retinal cells, nfo=Nerve fibers to optic nerve.

Western blot

Extract bands of *B. schaefferi* and *A. parthenogenetica* were marked with anti-p73 (65 kilodaltons) and anti-DBH (72k kilodaltons). The anti-DBH expression was similar in both anostraca species, whereas the intensity of the anti-p73 reaction was higher in *B. schaefferi* and *A. parthenogenetica* (Fig.6).

Discussion

The moveable eyestalks in adult *B. schaefferi* and *A. parthenogenetica* are comprised of the compound eye and two optic neuropils, the lamina ganglionaris and medulla externa [28]. Axon bundles emerging from the medulla externa form the optic nerve (ON), which targets the brain. Tangential horizontal sections of the compound eye reveal the structure of the crystalline cone, which is composed of four cells [12, 28] . In the dorsal-most sections, the lamina ganglionaris has an oblong shape and is orientated parallel to the base of the compound eye. A layer of neuronal cell bodies (about three to five somata wide) is located along the distal margin of the lamina [20, 28].

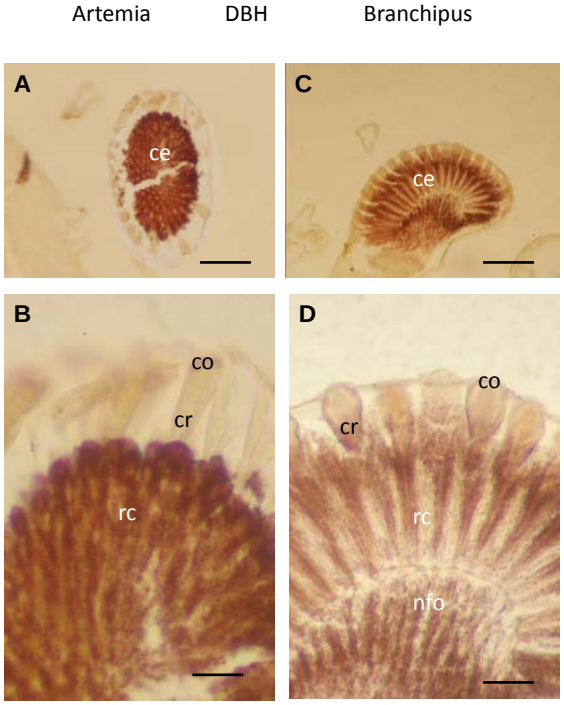

Figure 2.
Sagittal section of the compound eye (ce) of *A. parthenogenetica* and *B. schaefferi* marked with anti-dopamine beta hydroxylase (DBH). A, B, ArP; C,D BrS. Scale bars: 100 µm in A, C; 20 µm in B,C.
ce=compound eye, co=cornea, rc= retinal cells, nfo=Nerve fibers to optic nerve

The expression of DBH in diverse ocular tissues has been described in different animal species and humans [4, 24]. DBH immunoreaction product was found in the anterior segment in the peripheral corneal endothelium layer, in both the dilator and sphincter muscles of the iris, as well as in the anterior border layer of the iris and the ciliary muscle and the stroma of the ciliary processes. DBH staining, in the posterior segment, was seen around blood vessels in the choroid, in the vascular walls of the short posterior ciliary arteries and in the ciliary nerves. The retina was also immunopositive, with specific labeling in the rods and cones of photoreceptors, inner and outer plexiform layers and the ganglion cell layer. There is no significant difference in the distribution of DBH-related immunoreactivity in human and monkey eyes [4,24].

On the other hand, p73 is present as a truncated isoform in developing neurons and whose levels are dramatically decreased during sympathetic neuron apoptosis after nerve growth factor

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withdrawal [3, 22]. Therefore, p73 is necessary for the survival and long-term maintenance of central nervous system neurons, including the peripheral nervous system [3,21,22].

The retinal cells of the compound eye are considered as a part of the peripheral nervous system and p53, a family member of p73, could be a regulator during early ocular development and in the post-mitotic retina [27]. These results could be in agreement with the results found here which showed that the DBH-ir expression was similar in both anostraca species, since the DBH-ir was mostly located in the retinal cells of the compound eye. However, in the findings here, p73-ir expression was different in *B. schaefferi* and *A. parthenogenetica*. Hence, the question is, why is the p73 expression in the *B. schaefferi* mostly found in retinal cells and why is it mostly found around the crystalline and cornea in the *A. parthenogenetica* ? The explanation for why the p73 expression is in the retinal cells in *A. parthenogenetica* could be logical, because the retinal cells are where the DBH expression is present and where equilibrium between both p73 isoforms, TAp73 and ΔNp73 is described as being necessary for the survival and maintenance of the sympathetic neurons [3,17, 21, 22].

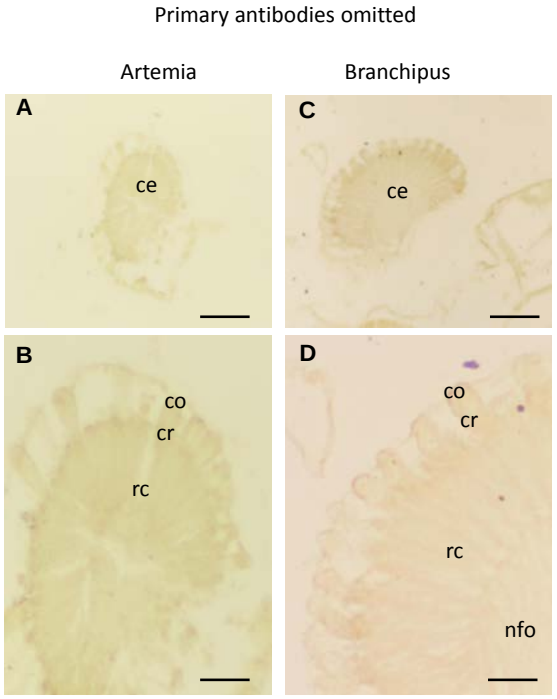

Figure 4.
Sagittal section of the compound eye (CE) of *A. parthenogenetica* A, B, and *B. Schaefferi* C,D. Method specificity, primary antibodies omitted. Scale bars: 100 µm in A, C; 20 µm in B,C. ce=compound eye, co=cornea, rc= retinal cells, nfo=Nerve fibers to optic nerve

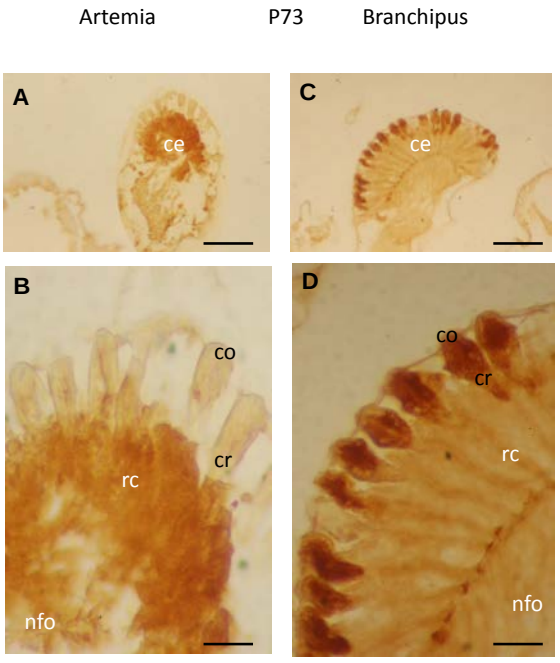

Figure 3.
Sagittal section of the compound eye (ce) of *A. parthenogenetica* and *B. schaefferi* marked with anti-TAp73 (p73). A, B, ArP; C,D BrS. Scale bars: 100 µm in A, C; 20 µm in B,C.
ce=compound eye, co=cornea, rc= retinal cells, nfo=Nerve fibers to optic nerve.

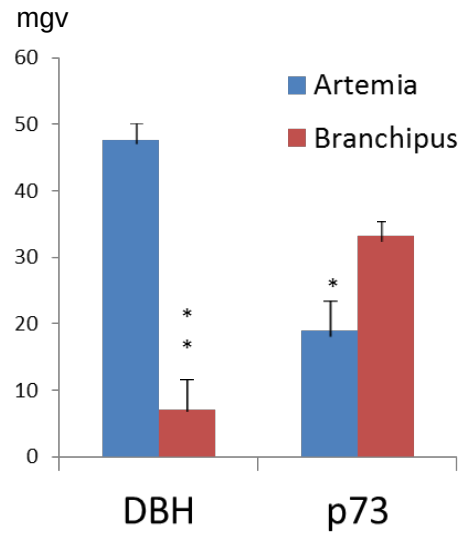

Figure 5.
Histogram of anti-p73 and anti-DBH immunohistochemistry expression measured (mean gray value) by densitometry in *A. parthenogenetica* and *B. schaefferi*. mgv= mean gray value. Significant differences: *=p>0.05, **=p>0.01

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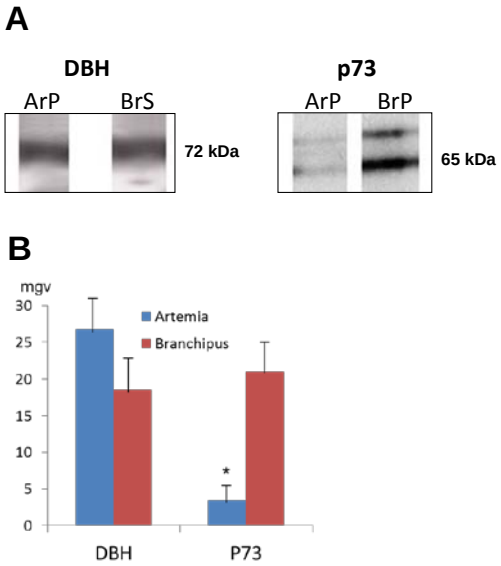

Figure 6.
Western-blot and Histogram of anti-p73 and anti-DBH expression measured (mean gray value) by densitometry in *A. parthenogenetica* (ArP) and *B. schaefferi* (BrS). mgv= mean gray value, kDa= kilodalton. Significant differences: *=p>0.05

Nevertheless, what could be the explanation for why p73 expression in the *B. schaefferi* is mainly localized around the crystalline and cornea, whereas DBH expression is located in retinal cells. One explanation could be the different habitats of both anostraca species, where p73 in the hypersaline water *A. parthenogenetica* plays the expected role for the survival of the peripheral nervous system, whereas p73 in fresh water *B. schaefferi* could also play a further role and/or function yet to be identified.

In conclusion, DBH and p73 are expressed in the eyes of both the anostraca species. DBH expression is mainly located in retinal cells of both species as occurs in nearly all animal eyes and p73 is expressed in the retinal cells of *A. parthenogenetica* eyes but p73 is mostly present around the crystalline and cornea in the *B. schaefferi* eyes. Therefore, further studies would be necessary to explain the differences in p73 and DBH expression in the eyes of these two anostraca species.

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