Journal of Chemical Neuroanatomy 41 (2011) 170–180

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

The pineal complex of the European sea bass (*Dicentrarchus labrax*): I. Histological, immunohistochemical and qPCR study

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ARTICLE INFO

Article history:
Received 1 December 2010
Received in revised form 28 January 2011
Accepted 28 January 2011
Available online 16 February 2011

Keywords:
Pineal
Melatonin
Opsin
Aanat2
Hiomt
Sea bass

ABSTRACT

The pineal organ of fish is a photosensory and neuroendocrine epithalamic structure that plays a key role in the temporal organisation of physiological and behavioural processes. In this study performed in the European sea bass, *Dicentrarchus labrax*, we provided an in-depth description of the macroscopic and microscopic anatomy of the pineal organ and identified the presence of photoreceptor and presumed melatonin-producing cells using histological and immunohistochemical techniques. In addition, we analysed in the pineal the day–night expression (using quantitative real-time PCR) of two key enzymes in the melatonin-synthesising pathway; arylalkylamine-*N*-acetyltransferase 2 (AANAT2) and hydroxyindole-*O*-methyltransferase (HIOMT). The pineal complex of sea bass consisted of a narrow and short pineal stalk that adopts a vertical disposition, a small-sized pineal end vesicle firmly attached to the skull by connective tissue, a parapineal organ and a convoluted dorsal sac. Immunohistochemical study showed the presence of abundant serotonin-positive cells. Cone opsin-like and rod opsin-like photoreceptor cells were also evidenced in the pineal stalk and vesicle. Both *Aanat2* and *Hiomt* were expressed in sea bass pineal organ. *Aanat2* exhibited higher nocturnal transcript levels, while no significant day–night differences were found for *Hiomt*. These results, together with ongoing studies analysing neural and neurohormonal outputs from the pineal organ of sea bass, provide the basic framework to understand the transduction integration of light stimulus in this relevant species for marine aquaculture.

1. Introduction

The pineal organ of teleosts is an epithalamic structure that has photosensory and neuroendocrine functions (Ekström and Meissl, 1997, 2003). This organ transforms the information derived from the light/dark cycle into neural (excitatory neurotransmitters) and hormonal (melatonin) rhythmic outputs (Collin et al., 1989; Falcón et al., 2010) that play a key role in the temporal organisation of relevant physiological and behavioural processes (Khan and Thomas, 1996; Vanecek, 1998; Wright, 2002; Falcón et al., 2003; Danilova et al., 2004; López-Olmeda et al., 2006; Ziv and Gothilf, 2006a). Using different rod and cone opsin specific antibodies, rod-like and cone-like photopigments have been identified within the pineal organ of different fish species (Vigh-Teichmann et al., 1983; Ekström et al., 1987; García-Fernández et al., 1997; Vigh et al., 1998, 2002; Foster and Hankins, 2002). In addition, the expression of novel opsins (green-like and ultraviolet-like cone opsins, pinopsin, vertebrate ancient-opsin, parapinopsin and parietopsin) has been described in the pineal complex of some teleost species (Fejér et al., 1997; Philp et al., 2000a,b; Forsell et al., 2001, 2002; Peirson et al., 2009); however, it seems that a rod-like opsin (exo-rhodopsin) that differs from that found in the lateral eyes is the predominant photopigment present in the fish pineal (Mano et al., 1999; Philp et al., 2000a; Vuilleumier et al., 2006; Peirson et al., 2009).

The neurohormonal information from the pineal organ is relayed by melatonin, the production of which provides daily, lunar and seasonal time information (Reiter, 1993; Oliveira et al., 2010; Falcón et al., 2010). While retinal melatonin seems to be an autocrine and/or paracrine factor that is metabolised locally, pineal melatonin is released rhythmically into the cerebrospinal fluid and blood, and acts on specific targets through melatonin receptors (Reppert, 1997; Falcón et al., 2010). In all vertebrates, melatonin

Abbreviations: *Aanat2*, arylalkylamine-*N*-acetyltransferase 2; *Hiomt*, hydroxyindole-*O*-methyltransferase; ZT, Zeitgeber Time; 5-HT, serotonin.

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doi:10.1016/j.jchemneu.2011.01.006

is synthesised from tryptophan, which is hydroxylated and decarboxylated leading to the formation of serotonin. In turn, serotonin is transformed into melatonin by the arylalkylamine *N*-acetyltransferase (AANAT) that catalyses the formation of *N*-acetylserotonin, and the hydroxyindole-*O*-methyltransferase (HIOMT) that converts the *N*-acetylserotonin formed into melatonin (Klein et al., 1997; Falcón, 1999; Klein, 2007). The rhythmic profile in melatonin secretion by the pineal reflects an increase in nocturnal AANAT transcription and/or activity, whereas *Hiomt* expression and activity does not exhibit marked variation throughout the light–dark cycle (Klein, 2007; Vuilleumier et al., 2007; Falcón et al., 2010). Unlike most vertebrates, two different *Aanat* genes (*Aanat1* and *Aanat2*) are present in teleost fish. Fish *Aanat1* is the orthologue of tetrapod *Aanats* and seems to have a more wide distribution in the retina and brain, whereas *Aanat2* is more specifically expressed in the pineal organ (Coon et al., 1999; Coon and Klein, 2006).

Previous studies have focused on characterising the function of the pineal organ in the European sea bass by analysing its light sensitivity, rhythmic *in vivo* and *in vitro* melatonin secretion and targets (Iigo et al., 1997; Sánchez-Vázquez et al., 1997; García-Allegue et al., 2001; Bayarri et al., 2002, 2003, 2004a,b,c; Migaud et al., 2006, 2007); however, the anatomical, ultrastructural and molecular characteristics of the pineal organ of this species have never been explored in depth. Recently, we have reported the neuroanatomical and functional links of the pineal organ with GnRH2 system in the European sea bass (Servili et al., 2010) and the distribution of melatonin receptors in the brain of this species (Herrera-Pérez et al., 2010). In this study, we present an in depth-description of the anatomy and morpho-functional characteristics of the pineal organ, with special attention being paid to the identification of the photoreceptor and melatonin producing-cells, by means of histological and immunohistochemical techniques. Furthermore, we also investigated the day–night expression patterns of two key enzymes of the melatonin-synthesising pathway, the arylalkylamine-*N*-acetyltransferase 2 (AANAT2) and hydroxyindole-*O*-methyltransferase (HIOMT), analysed by quantitative real-time PCR.

2. Materials and methods

2.1. Animals

Juvenile specimens of sea bass (14–19 cm in length, 40–100 g in body mass) were used for the anatomical, histological and immunohistochemical studies. For the quantitative real-time PCR study, adult sea bass male specimens of 2–2.5 kg in body mass were analysed. Animals were purchased from a local fishery (Cupimar, San Fernando, Spain) and kept in running seawater at constant temperature and salinity (19 ± 1 °C and 39 ppt, respectively) in indoor facilities from the "Laboratorio de Cultivos Marinos" (University of Cadiz, Puerto Real, Spain) receiving natural environmental light. For histological and immunohistochemical analysis, all animals were processed in October (sunrise 07:27 h; sunset, 18:56 h, GMT + 1) between 10.00 h and 14.00 h (local time, GMT + 1). To determine day–night variations in pineal *Aanat2* and *Hiomt* mRNA expression a total of 7 adult animals were sampled in October at two different Zeitgeber Time (ZT) points: ZT5 (12:30 h GMT + 1, $n = 4$) and ZT17 (00:30 h GMT + 1, $n = 3$). All animals were treated in agreement with the European Union regulation (EC Directive 86/609/EEC) concerning the protection of experimental animals. Animal experimental protocols were approved by the Animal Care and Use Committee of the University of Cadiz.

2.2. Histological study

Twelve animals were anesthetized in MS-222 (Sigma–Aldrich, St. Louis, MO) and perfused via the aortic bulb with 0.6% saline solution followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). In order to preserve the pineal position, the brain and pineal organ were not excised and whole heads were processed using a decalcification protocol of the cranium bones. The heads were immersed in a solution containing sodium citrate (50%, w/v) and formic acid (50%, v/v) at a 1:1 proportion. Decalcifying solution was changed every 24 h until the bones were soft enough to be sectioned. Whole heads were then dehydrated, embedded in paraffin and sectioned in coronal ($n = 6$) and sagittal planes ($n = 6$). The 10 μm-thick sections obtained were stained with hematoxylin-VOF (light green, orange G, acidic fuchsine), analysed on an Olympus photomicroscope BH2 (Olympus, Tokyo, Japan) and photographed with an Olympus C5050 zoom Digital Camera, 5.0 MP (Olympus, Tokyo, Japan).

2.3. Immunohistochemical study

Eight animals were anesthetized in MS-222 (Sigma–Aldrich) and perfused via the aortic bulb with 0.6% saline solution followed by Bouin's fixative (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4). The brains with the pineal organ attached were then removed and post-fixed in the same fixative for 5–7 h in the dark at 4 °C. After cryoprotection overnight in 0.1 M phosphate buffer containing 15% sucrose, brains were embedded in Tissue-Tek, frozen in cold isopentane, and serial coronal ($n = 4$) and sagittal ($n = 4$) sections of 14 μm-thick were obtained with a cryostat. Immunohistochemical staining was performed using immunofluorescence and immunoperoxidase methods. Before immunostaining, sections were transferred to Coons buffer (0.01 M Veronal, 0.15 M NaCl) containing 0.1–0.2% Triton X-100 (CBT) for 10 min and then saturated in CBT containing 0.5% casein for 30 min. Sections were incubated overnight at room temperature in a moist chamber with a rabbit anti-bovine rod opsin (1:500 dilution, CERN-922, source Dr. Willem J. De Grip), human LW cone opsin (1:500 dilution, CERN-874, source Dr. Willem J. De Grip) and serotonin (1:1000 dilution, source Dr. H.W. Steinbusch). The antibodies used in this study have previously been well characterised and used successfully in fish to identify opsins (García-Fernández et al., 1997; Confente et al., 2008) and serotonin (Steinbusch et al., 1983; Falcón et al., 1984). For immunofluorescence methods, sections were washed in CBT (3× 15 min) and incubated for 2 h at room temperature with an antibody against rabbit IgG coupled to fluorescein or Texas Red (1:100 dilution, Jackson ImmunoResearch, West Grove, USA). After washing in CBT (3× 15 min), sections were mounted with Vectashield (Vector Laboratories, Burlingame, USA). For immunoperoxidase methods, after incubation with primary antibody sections were washed in CBT (2× 15 min) and incubated for 1.5 h at room temperature with Biotin-sp-Conjugated-AffiniPure Goat Anti-rabbit-IgG (Jackson Immuno Research Laboratories Inc.) diluted 1:1000 in CBT. After washing in CBT (2× 15 min), sections were incubated 1.5 h at room temperature with peroxidase-conjugated-streptavidin complex (Jackson Immuno Research Laboratories Inc.) diluted 1:1000 in CBT. Finally, sections were washed in CBT followed by Tris–HCl (0.05 M, pH 7.4) and peroxidase activity was visualised either in 0.05 M Tris–HCl, pH 7.6 containing 0.025% 3,3-diaminobenzidine tetrahydrochloride (Sigma–Aldrich) and 0.01% hydrogen peroxide or 0.04% 4-chloro-1-naphthol (Sigma–Aldrich) and 0.01% hydrogen peroxide. Negative controls were performed by replacing the primary or secondary antisera with normal non-immune rabbit serum and omission of primary or biotinylated antisera. The immunostaining was analysed on an Olympus photomicroscope BH2 (Olympus, Tokyo, Japan) provided with adequate fluorescence filters and microphotographs were obtained with an Olympus C5050 zoom Digital Camera, 5.0 MP (Olympus).

2.4. Quantitative real-time PCR study

The pineal organs of adult sea bass specimens were excised and quickly frozen in liquid nitrogen. Total RNA was extracted individually from pineals using "EUROzol" (EuroClone, Siziano, Italy) according to the manufacturer's instructions. Total RNA (1 μg) was retro-transcribed using a kit that eliminates genomic DNA contaminations (QuantiTect® Reverse Transcription Kit, Quiagen, Hilden, Germany). Real time gene expression analysis was performed in a Chromo 4™ Four-Color Real-Time System (Biorad, Alcobendas, Spain) using L17 for normalisation (GenBank accession number AF139590). PCR reactions were developed in duplicate in a 25 μl volume using cDNA generated from 1 μg of RNA, iTaqTM SYBR® Green Supermix with ROX (Biorad) and specific primers (0.4 μM, Table 1). Standard curves were generated with serial dilutions of cDNA transcribed from 1 μg of total RNA. All calibration curves exhibited slopes close to −3.32 and efficiencies around 100%. The conditions of the PCR reactions were very similar for the three genes analysed: 3 min at 95 °C, 30 s at 95 °C, 45 s at 52 °C (for *Hiomt*) or 56 °C (for *Aanat2* and *L17*), and 45 s at 72 °C. The number of cycles was 30 for *L17* and 38 for *Aanat2* and *Hiomt*. PCR products obtained were run in agarose gels and sequenced to ensure the specificity of the amplification. Besides, melting curves were analysed for each sample, in order to confirm that only a single sequence was amplified. Negative controls included replacement of cDNA by water and the use of non retro-transcribed total RNA. The ΔΔCt method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression. Day–night statistical differences in expression were determined using the *T-Student* test.

Table 1
Specific primers pairs used for quantitative real-time PCR analysis in the pineal organ of the European sea bass.

Primers	Forward	Reverse
AANAT2	5′TGAGGAGGGACAGCTGGTAG3′	5′TCGCCTCCTGTGAAAGTCTC3′
HIOMT	5′CTGGGAAGATTTACGATGCAG3′	5′TCAGTCCTTGGACTTGTGTACC3′
L17	5′TGATACGGCAGCGGAAGTC3′	5′GACTCCTGCGTTGTCTTCAAA3′