Comparative Biochemistry and Physiology, Part A 185 (2015) 69–79

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Melatonin-induced changes in *kiss*/*gnrh* gene expression patterns in the brain of male sea bass during spermatogenesis

María Victoria Alvarado, Manuel Carrillo, Alicia Felip *

Consejo Superior de Investigaciones Científicas (CSIC), Instituto de Acuicultura de Torre de la Sal, s/n. 12595 Ribera de Cabanes, Castellón, Spain

ARTICLE INFO

Article history:
Received 22 January 2015
Received in revised form 11 March 2015
Accepted 16 March 2015
Available online 22 March 2015

Keywords:
GnRH/gonadotropins
Growth
Kisspeptins
Maturation
Melatonin implants
Reproductive hormones
Seasonal variations
Teleost

ABSTRACT

Evidence exists that melatonin may drive the seasonal changes in kisspeptin-expressing cells and GnRH/gonadotropin secretion in mammals, thus modulating their reproductive activity. This study established the influence of long-term melatonin administration (as an implant) on growth performance and reproduction of adult male sea bass. Melatonin reduced the fish weight and condition factor, thus affecting the performance of fish. Melatonin also affected gonadogenesis, as shown by a decrease in the gonadosomatic index after 150 days of treatment and the lower percentage of running males during the spermatogenesis and full spermiation stages of this species. Exogenous melatonin also resulted in lower plasma androgen levels during the reproductive period, and showed a significant decrease in serum Lh and Fsh concentration after 30 and 60 days of treatment, respectively. Thus, melatonin elicited seasonal changes in key reproductive hormones that affected testicular maturity. The hypothalamic expression of *kiss1* was significantly higher in melatonin-treated fish than in controls after 30 days of treatment, while a significant increase in *kiss2* expression was detected on day 90 of treatment. By contrast, melatonin showed a significant decrease in kisspeptin expression in the dorsal brain on day 150 of treatment and also affected the expression of *gnrh-1* and *gnrh-3* and *gnrhr-II-1a* and *2b* and the *fshβ* gene in the pituitary. These results suggest that in this species, melatonin evokes changes in the mRNA levels of kisspeptin and gnrh system genes that appear to mirror disturbances in spermatogenesis.

1. Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine synthesized by pineal gland and retinal photoreceptors and secreted in a circadian manner during the night in all the vertebrates studied so far. This molecule acts as a neuroendocrine signal and plays a major role in several time-regulated functions, such as food intake, growth, immune response, osmoregulation and reproduction, among others (Simonneaux et al., 2009; Falcón et al., 2010). There is evidence demonstrating that the control of reproduction by melatonin is species-specific. Although the mechanisms of action of melatonin on the hypothalamus-pituitary-gonad (HPG) axis remain to be determined, the presence of melatonin receptors in distinct nuclei of the brain, pituitary and gonads suggests that melatonin has multiple sites of action in the endocrine control of this process (Dubocovich et al., 2003; Falcón et al., 2010).

In mammals, melatonin may drive seasonal changes in kisspeptin and GnRH expressing cells and, in turn, mediates reproductive events (Simonneaux et al., 2009). Thus, in seasonal breeders such as hamsters, a short photoperiod induces gonadal regression being the HPG axis reactivated after kisspeptin administration although differences can exist according to the sex within the same species (Greives et al., 2007; Mason et al., 2007; Ansel et al., 2011). The influences of melatonin on several daily and annual processes have been also widely investigated in teleost fish through the use of photoperiod manipulations, pinealectomy and/or melatonin administration (Falcón et al., 2010). In this context, there is considerable evidence for melatonin involvement in the gonadotropin secretion and gonadal maturity (Khan and Thomas, 1996; Amano et al., 2004; Singh et al., 2012), as well as it seems apparent that melatonin influences growth and feeding (Rubio et al., 2004; De Pedro et al., 2008; Singh et al., 2012) and lipid and protein metabolism (Singh et al., 2012) in fish. However, very few studies have explored the neuronal networks through which melatonin acts to influence the activity of the reproductive axis. Recent studies have reported that exposure to different doses of melatonin of female zebrafish (*Danio rerio*) induces a dose-related increase of *kiss1*, *kiss2* and *gnrh3* gene expression in the brain and of *lhβ* in the pituitary suggesting that melatonin promotes reproduction in this species (Carnevali et al., 2011). By contrast, the use of implants containing melatonin in European eel (*Anguilla anguilla*) had no effects on *gnrh* RNA expression, but decreased both gonadotropin mRNA expression and steroid plasma levels, thus inhibiting its reproductive function (Sébert et al., 2008). It has been

* Corresponding author. Tel.: +34 964 319500; fax: +34 964 319509.
E-mail addresses: v.alvaradoferdz@gmail.com (M.V. Alvarado), manuelcarrilloestevez@gmail.com (M. Carrillo), afelip@iats.csic.es (A. Felip).

http://dx.doi.org/10.1016/j.cbpa.2015.03.010
1095-6433/

suggested that the effects of melatonin in orange-spotted grouper (*Epinephelus coioides*) may be mediated by MT1 receptors, as a decrease in MT1 expression would increase the expression of *gnrh-1* by upregulating *kiss2* expression (Chai et al., 2013).

European sea bass is a highly valued fish that, in the Mediterranean area, reproduces under short photoperiod regimes in the winter (December–March) and ceases reproduction in the spring, when the length of the day increases (Carrillo et al., 1995, 2009). It is known that photoperiod manipulation affects both the daily and seasonal rhythms of melatonin and reproductive hormones in this species, thus influencing sexual maturation (García-Allegue et al., 2001; Bayarri et al., 2004, 2009, 2010; Rodríguez et al., 2004; Felip et al., 2008). Furthermore, melatonin administration (as an injection in mid-March) has been shown to inhibit nocturnal mRNA expression of *gnrh-1*, *gnrh-3* and gnrh receptors (Servili et al., 2013), suggesting that distinct neuronal systems might mediate photoperiodic changes and influence its reproduction. It is known that the sea bass has two distinct *kiss* genes and two kiss receptors, being the Kiss2 decapeptide more potent than the Kiss1 peptide in inducing gonadotropin secretion after systemic injection in prepubertal fish (Felip et al., 2009; Tena-Sempere et al., 2012). Additionally, changes in kisspeptin expression in the brain of adult fish in this species during different gonadal stages suggest a potential implication of kisspeptins in the seasonal control of its reproduction (Migaud et al., 2012; Alvarado et al., 2013). In this context, seasonally-breeding fish, such as sea bass, provide an ideal model for studying the neuroendocrine systems responsible for integrating environmental information in the reproductive axis. In this work, we investigated the brain expression changes of these elements of the kisspeptin and gnrh systems including the two kisspeptin genes (*kiss1*, *kiss2*) and their receptors (*gpr54-1b*, *gpr54-2b*) as well as *gnrh-1*, *gnrh-2*, *gnrh-3* and *gnrh-II-1a* and *gnrh-II-2b* and gonadotropin genes (*fshβ*, *lhβ*), as they are known to be involved in the neuroendocrine control of animal reproduction (Oakley et al., 2009; Zohar et al., 2010), in response to long-term melatonin administration, as well as its potential effects on the activity of the pituitary-gonad axis and certain biometric parameters and gonadal maturity in male sea bass.

2. Material and methods

2.1. Animal housing

Fish were purchased from Baseviva (Sant Pere Pescador, Gerona, Spain) and maintained at the Instituto de Acuicultura de Torre de la Sal (IATS; Castellón, Spain, 40°N 0°E) facilities, where the experiment was carried out. Seventy 2-year-old male sea bass with a body weight of 144.82 ± 5.56 g and a length of 22.96 ± 0.23 cm were distributed into separate identical 500 L fiberglass tanks, provided with well-aerated running water (salinity = 37–38‰). Temperature (12-25 ± 1 °C) and photoperiod followed the variations recorded in the natural environment. Fish were fed until apparent satiety twice a day, using pellets from Proaqua Nutrición, S.A. (Palencia, Spain) (protein 54–45%, lipids 20–12%, carbohydrates 9–25%, ash 11%, moisture 1–3%, DE 22.4-19.7 MJ kg^{-1}).

2.2. Preparation of melatonin implants

Melatonin was purchased from Sigma (St. Louis, MO), and solid silastic implants (DowCorning, Midland, MI) were prepared as previously described for sea bass (Zanuy et al., 1999). The exogenous melatonin dose used in this study is based on previous studies carried out in other fish species (Sébert et al., 2008). Fish were deeply anesthetized with 2-phenoxyethanol and implants were administered via a small 2- to 3-mm incision in the abdomen. All fish were treated with povidone iodine (Betadine®) after implantation. All animal experiments were conducted in accordance with the guidelines for animal experimentation established by European legislation (ETS No. 123, 01/01/91).

2.3. Experimental design and sampling

Fish were organized into two groups (35 fish each): in the first, which acted as a control group (sham implant, C), the animals were administered empty implants, whereas in the second, the animals were administered melatonin implants (M). Fish received a first implant, either empty or containing a melatonin dose of 3.81 mg/g of fish body weight, in October (day 0, the start of the experiment), coinciding with the early testicular recrudescence (gonadosomatic index of 0.05 ± 0.01%) of male sea bass (Carrillo et al., 1995), and then a second identical implant 60 days later (December). The time chart of experimental procedure is described in Fig. 1. Accordingly, from October to April, all fish were periodically weighed (W) and their length was measured (L). Fulton's condition factor (K) as well as the specific growth rates for weight (SGR_W) and length (SGR_L) and biometric indexes including the gonadosomatic (GSI), hepatosomatic (HSI) and mesenteric fat indexes (MFI) were calculated following the biometric procedure and formula used by Felip et al. (2008). Gonads were collected for histological analysis (Bennett et al., 1976; McDowell and Trump, 1976) and the stages for testicular development were assessed (Begtashi et al., 2004) using light microscopy (Jenamed 2). Images were taken using a Jenoptik (Germany) digital camera and processed with the Progres® CapturePro software and Photoshop CS5 (Adobe Systems, San Jose CA). The percentage of maturing males was periodically evaluated by applying gentle hand pressure to the abdomen of all fish during each sampling interval from January to April. Blood samples were collected during the light phase 0900–1100 h from the caudal vein. Plasma was separated by centrifugation at 4 °C and stored at −20 °C until analyzed. For tissue collection, the pituitary was separated from the brain, and the brain was dissected in order to remove the hypothalamus and dorsal brain, which included the whole olfactory bulb, telencephalon and optic tectum. It has been shown that kisspeptin (Escobar et al., 2013a) and gnrh (González-Martínez et al., 2002) neurons are distributed in these regions of the sea bass brain and more recently the telencephalon and optic tectum have been demonstrated to play a central role in the reproductive system in this species (Espigares et al., in press). All tissues were frozen on dry ice and stored at −80 °C until later use for total RNA extraction.

2.4. Enzyme immunoassays (EIA) of plasma hormonal levels

Plasma melatonin levels were measured using a commercial ELISA kit (IBL, Hamburg, Germany) (Bayarri et al., 2002). Plasma sex steroid levels were measured by conventional EIA, according to Rodríguez et al. (2000) for testosterone (T), Rodríguez et al. (2001) for 11-ketotestosterone (11-Kt), Molés et al. (2008) for 17β-estradiol (E_2), Mateos et al. (2006) for luteinizing hormone (Lh) and Molés et al. (2012) for follicle-stimulating hormone (Fsh).

2.5. RNA isolation and reverse transcription for quantitative real-time PCR (qRT-PCR)

The procedure for the RNA isolation and reverse transcription for qRT-PCR was carried out as previously described (Alvarado et al., 2013). Gene-specific primers and Taqman fluorogenic probes (when necessary) used in this study for qRT-PCR analysis are described in Table S1. The sea bass elongation factor-1α (*ef1α*) gene was used as control gene as previously reported (Alvarado et al., 2013). Data were expressed as relative values of mRNA for each target gene/mRNA *ef1α* (starting quantity mean ± standard error of the mean, SEM). Negative controls were also run for each real-time experiment.